BACTERIA-BASED MOLECULAR ASSAY DETECTION SYSTEM

[B-MAD]

by

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A Thesis submitted to the Faculty of

THE BIOMEDICAL ENGINEERING GIDP

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN BIOMEDICAL ENGINEERING
In the Graduate College

THE UNIVERSITY OF ARIZONA

December 2005

Statement by Author

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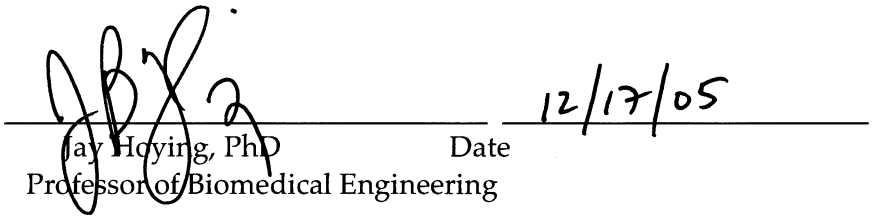

12/17/05

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Abstract

Bacteria possess natural mechanisms allowing them to adapt to the environment and communicate with each other, processing large amounts of information in parallel. Cyanobacteria survive in a variety of environments and respond to light. Vibrio harveyi, a marine bacteria, and Pseudomonas aureofaciens, a wheat bacteria, communicate using small molecules; V. harveyi bioluminesces in response to an inter-species signaling molecule, while P. aureofaciens produces phenazine in response to its intra-species signaling molecule. These inherent signaling mechanisms can be engineered to create rapid, specific, modular Bacteria-Based Molecular Assay Detection (B-MAD) systems. A B-MAD system designed to detect the human pathogen Clostridium perfringens serves as proof of concept for completely biological information processing units. The B-MAD system consists of three engineered cyanobacteria cells used in combination to detect Clostridium perfringens, a causative agent in gas gangrene, food poisoning and antibiotic-associated diarrhea. The B-MAD system is activated by blue/UV-A light and responds to AI-2, a small molecule produced by C. perfringens and perfringolysin O, a C. perfringens pore-forming toxin. It is possible, using the yellow fluorescence and bioluminescence outputs from the B-MAD system, to unambiguously detect C. perfringens. The design of the B-MAD system as well as the fabrication of components for the Blue Light Converter are reported.

Introduction and Background

Opening

Bacteria naturally respond to changes in environmental factors, including temperature, pH, and light. To protect themselves against changes which pose a danger to the bacteria, they posses the ability to move using flagella, sporulate in extreme conditions (gram positive bacteria only) and translate proteins to protect themselves from harmful UV rays. Some bacteria release quorum sensing signals detectable by various other species of bacteria; these signals allow the individual bacteria to sense total bacterial numbers present and trigger the production of virulence factors to help compete for resources in places with a large bacterial load. All of these methods are carefully controlled by the bacteria, responding quickly to minute changes in the environment. The sensitivity, precision and capability to process and respond to a multitude of inputs simultaneously make bacteria extremely powerful and efficient data processors when compared to today's computer processing standards. Thus, researchers are beginning to explore the possibility of bacteria and their components as models for simple digital logic gates and parallel processors. While most of the current research evaluates the possibility of developing "DNA computers," there is the opportunity to use engineered bacteria themselves as sensors and computer processing components.

In contrast to building computational systems within a single cell, few experimental endeavors have focused on constructing computational networks involving mul-

tiple cells. Some computer engineering groups have presented models and theory of intercellular computational networks, but much of this research has yet to be carried out in the lab. Of the many researchers who have closely studied and characterized bacteria cellular communication, both intra-species and inter-species, these groups have focused primarily on the task of completely understanding and identifying all components involved in the complicated biological signaling cascades. The information uncovered by these research groups serves as the backbone for developing engineered bacterial communicating networks. Such an intercellular network built through synthetic biology, engineering the genetic elements of a cell to produce a specific, planned action in the resulting cell, would have widespread applications.

A biological intercellular computer has the potential to become a powerful new technology for researchers, industry and the military. Networks of cells completing computation and data analysis will allow scientists, doctors and engineers to tackle new, complex problems requiring unique, flexible computing capabilities. Biological computing networks could conceivably one day be used inside the human body to attack cancers, supply insulin, locate tumors and identify the difference between malignant and benign ones, monitor kidney function and detect infectious diseases. These biologically-based computers will make it easier to interface traditional computing networks directly with biological molecules, such as insulin or botulinum toxin, without complicated, expensive traditional detection and computational equipment. This simple interface is par-

ticularly useful in space-limited technology applications like those found on cruise ships, forward military hospitals or even on the space shuttle. The potential of developing biological computers is only now becoming apparent, as DNA tecnology provides researchers the power to freely alter the genes within a cell. While some have discussed the possibility of these biological computers, few have developed any type of purposely engineered intercellular network for practical clinical or industrial applications.

Currently, few detectors exist for Clostridium perfringens. Today, more and more researchers are evaluating methods to detect C. perfringens due in large part to its potential use as a bioterror agent. One patented detection method for Clostridium perfringens (pub. No. 20060028644, type A1) relies on the Raman light scattering of Clostridium perfringens for detection. This system also claims to be able to distinguish a wide variety of pathogenic microorganisms, including Bacillus anthracis, the Anthrax producing bacteria. Another commonly proposed detection method, although not fully defined, includes utilizing mixed PCR assays to identify microorganisms, specifically C. perfringens. The lack of a reliable, inexpensive Clostridium perfringens detector system led the U.S. government to fund "Project Zebra," where researchers work to develop a mechanism to detect pathogens such as C. perfringens based on the gene response differences that occur in the presence of these pathogens. At this time, none of these C. perfringens detection systems are commercially used.

Traditional detectors for biological molecules rely on interfacing computers and other modern technology with biological systems. These systems work on the principles of the mechanical, chemical and electrical components of the specific artificial probe used in each application. By far, the greatest challenge for these sensing devices is interfacing with the biological systems. More modern detection systems, such as ELISA (Enzyme-Linked Immunosorbent Assay) tests, where antibodies are used as detectors, incorporate a biological molecule to specifically detect a target molecule. These systems also have drawbacks; antibodies for these tests can be difficult to produce and take time to engineer, and the complexity of many infectious diseases make it difficult to identify specific etiologies with only one ELISA test. Rather, an array of tests together allow clinicians to diagnose many infectious diseases. Constructing a detection system that uses natural biological sensing mechanisms would allow the engineered bacteria to sense the environment, processing multiple signals, molecules and environmental conditions (light, pH, etc...) and make a determination of disease etiology based on the array of data collected by the bacterial system. Bacteria's ability to process data in parallel make a bacteria-based detection system advantageous in the clinic, as the bacterial system would perform the function of multiple traditional tests as well as the determination of etiology based on the data from the array of tests. The B-MAD system requires no electrical power, as it is made up of cyanobacteria, which feed on light and nutrients in the environment. These systems have the potential to add specificity to the diagnosis of dis-

eases and streamline the diagnostic process, servings as unambiguous sensors rather than simple tests.

Background - Natural Intercellular Signaling Systems

For years, biologists have evaluated bacterial signaling mechanisms, both intra and inter-species. Perhaps the most well-known type of naturally occurring bacterial communication networks are "quorum sensing" systems, where bacteria send and receive signals indicating overall bacteria numbers. These signals can transcend species or be specific to only one species and have been identified in bacteria living in a wide variety of environments, from harmless marine bacteria to plant pathogens and even human pathogens such as Vibrio cholera. The intercellular communication of quorum sensing can be used to engineer networks of common bacteria. Two bacteria containing quorum sensing systems that are useful to designing engineered bacterial communication networks are Pseudomonas aureofaciens and Vibrio harveyi.

One of the most well-studied quorum sensing systems occurs in Vibrio harveyi, an aerobic, gram-negative, marine bacterium. Vibrio harveyi is not a human pathogen, but its quorum sensing systems are analogous to those found in the human pathogen Vibrio cholera; the quorum sensing system in V. cholera controls virulence genes [1], while those in V. harveyi regulate bioluminescence. Therefore, researchers have worked to better understand the Vibrio quorum sensing systems in an effort to develop new treatments for cholera infections. Originally thought to have two parallel quorum sensing systems, recent research has identified a third quorum sensing system in V. harveyi [2]. The quorum sensing systems in V. harveyi regulate a variety of genes, including bio-

luminescence genes and are controlled by the quorum sensing signals HAI-1, AI-2 and CAI-1[2]. System one is regulated by HAI-1 and experimental evidence indicates that the signal is restricted to signal among V. harveyi [3]. System two is regulated by the small molecule, AI-2, which has the capability to stimulate a wide variety of bacteria, both gram-positive and gram-negative, and regulate an array of genes in the target bacteria [4]. Initial studies linked the production of AI-2 to the luxS gene. The luxS sequence was then used to query databases at the National Center for Biotechnology Information web-site; there were 58 different bacteria identified containing the luxS gene [5]. As there are innumerable numbers of bacteria yet unidentified and un-sequenced, there will clearly be more bacteria identified that contain the luxS gene. As investigators evaluated the genes and functions controlled by luxS in the known bacteria, there was a link, although not universal, to virulence genes and toxins in some of the most common pathogens plaguing humanity. For example, bacteremic infection of Neisseria meningitidis was linked to regulation by luxS and AI-2 [6]. Cholera toxin production and toxin-coregulated pilus are regulated by luxS and AI-2 in Vibrio cholera [7]. Secreted protease hemolysin in the common Streptococcus pyogenes infectious bacteria are controlled by luxS [8]. The alpha, kappa and theta toxins of Clostridium perfringens are also regulated by luxS and AI-2 [9]. In Campylobacter jejuni, a major food poisoning etiology, luxS was shown to control motility [10]. The specific functions, genes and mechanisms of AI-2 control are still being uncovered. The examples listed above are only a small sampling of the widespread control that AI-2 has over bacteria. Clearly AI-2 and other similar signal-

ing molecules play a major role in the pathogenesis of many bacteria. Careful analysis of the well-characterized quorum sensing signaling cascade regulated by AI-2 provides an opportunity to engineer the AI-2 signaling system to work to the advantage of researchers, clinicians and engineers.

In general, there are two types of quorum sensing signals, one used most often by gram-negative bacteria and the other most commonly found in gram-positive bacteria. Gram-positive bacteria, such as Staphylococcus aureus, utilize synthesized oligopeptides modified at specific locations and are detected by a response regulator protein that binds to DNA and regulates transcription[5]. Gram-negative bacteria, such as Vibrio harveyi or E. coli, most often synthesize acyl-homoserine lactone (AHL) autoinducers, which are able to freely diffuse across the bacteria cell membrane into the environment. These AHL's are detected by a specific class of proteins (LuxR-type), which in turn regulate gene transcription by binding specific DNA promoters [5]. AI-2, as well as the system one autoinducer, HAI-1, and the more recently identified system three autoinducer, CAI-1, are AHLs. To be able to engineer these bacterial communication systems, specifically the AI-2 production and response system, it is crucial to understand their natural mechanism of action within the cell.

Central to using AI-2 as an engineered signaling molecule is understanding the AI-2 production mechanism in V. harveyi and its production in a variety of other bacterial species. The initial substrate for AI-2 is S-adenosylmethionine (SAM), and the proc-

ess of synthesizing AI-2 relies on three enymatic steps [11]. In the synthesis of AI-2, SAM is converted to S-adenosylhomocysteine (SAH) when SAM is used in DNA synthesis, RNA synthesis or protein synthesis, which is a toxic intermediate that is hydrolyzed to S-ribosylhomocysteine (SRH) by 5'methylthioadenosine/S-adenosylhomocysteine (Pfs), a nucleoside enzyme [11]. Then, luxS is the catalyst in cleaving SRH, forming 4,5-dihydroxy 2,3-pentanedione (DPD), which cyclizes into furanone rings; one of those rings is the precursor to AI-2 [4]. AI-2's structure was eventually determined using crystallography and shown to be a novel furanosyl borate diester [12]. As SAM is present in bacteria, as is Pfs, the addition of the luxS gene to bacteria would give it the potential to synthesize AI-2.

In V. harveyi, AI-2 controls bioluminescence [13], Type III secretion systems (TTS) [14], polysaccharide [15] and metellaprotease [16] production. The AI-2 signaling cascades consists of 5 major proteins, small regulatory RNAs and Hfq, an RNA chaperone [17]. LuxP, the trans-membrane AI-2 receptor, LuxQ, an autophosphorolating protein, LuxU, a phosphotransferase protein, LuxO, a response regulatory protein, and LuxR, a transcriptional regulator, make up the AI-2 signaling cascade [2]. LuxR positively regulates the luxCDABE promoter that activates the transcription of the bioluminescence gene. LuxR also negatively regulates the expression of TTS genes [14]. The main area of interest in the AI-2 signal cascade is the regulation of bioluminescence, as it will be useful in engineering visual responses to AI-2 in cellular networks. In the absence of AI-2,

LuxP sits inactive across the inner cellular membrane, reaching into the periplasmic space. Meanwhile, with no active LuxP, LuxQ, which phosphorolates LuxU and passes the phosphate to LuxO [18]. The phosphorolated and active LuxO activates small regulatory RNAs that work to destablize the luxR mRNA [2]. Experimental evidence indicates that LuxR is required for the transcription of the bioluminescence genes, so there is no bioluminescence without LuxR; therefore, with luxO active and inhibiting luxR mRNA, there is no bioluminescence [19]. Macroscopically, when LuxP is not bound to AI-2, the cascade inhibits bioluminescence. Conversely, when there is AI-2 bound to LuxP, luxQ acts as a phosphatase instead of a kinase, which ultimately removes the phosphate from luxO, rendering it inactive and unable to inhibit the expression of luxR [18]. Therefore, in the presence of AI-2, the signaling cascade permits the expression of LuxR and, subsequently, the expression of bioluminescent genes and control over other LuxR-regulated genes. Focusing on the bioluminescent respose of V. harveyi to the presence and absence of AI-2 demonstrates a simple biologically controlled signaling system incorporating both inputs and outputs. Experimentation has identified the bioluminescent response of V. harveyi to AI-2 and other autoinducers. Among the autoinducers, AI-2 has a distinct, strong response independent of the other two AHL signaling systems found in V. harveyi [20]. The AI-2 signaling cascade is a well-characterized and studied signaling system to utilize in engineered cellular communication networks.

Pseudomonas aureofaciens strain 30-84, a gram-negative bacteria with the ability to suppress other bacterial disease of wheat, also has a characterized quorum-sensing system based on an AHL signal. The bacteria produces three phenazine antibiotics that suppress diseases of wheat [21] and similar to V. harveyi, has multiple identified intercellular signaling systems. One of those two signaling systems, the PhzR-PhZI quorum sensing system, plays an integral role in the production and regulation of phenazine similar to the controlled expression of bioluminescence in V. harveyi [22]. Unlike V. harveyi, however, the PhzR-PhzI signaling system in P. aureofaciens is controlled through only a single responsive protein, PhzR, instead of a signaling cascade [23]. The AHL signal in P. aureofaciens is hexanoylhomoserine lactone (HHL or C-6-HSL), which, like other members of the AHL family of signals and autoinducers, freely diffuses across the cellular membrane. Once in the cell, C-6-HSL binds and activates PhzR, a transcriptional regulator of the PhzR operon that, in P. aureofaciens, controls the expression of phenazine synthesis genes [22]. In the absence of the autoinducer C-6-HSL, PhzR is inactive and does not act as a transcriptional regulator. The autoinducer C-6-HSL is produced in P. aureofaciens via the expression of the phzI gene, which encodes an enzyme that catalyzes the synthesis of C-6-HSL [22]. In contrast to the action of many AI-2 controlled functions, the function controlled by quorum sensing in P. aureofaciens has a protective effect for its natural habitat, wheat roots, displaying the wide variety of cellular processes that quorum sensing controls in bacteria. The PhzR-PhzI quorum sensing system

in P. aureofaciens provides a second, well-defined, signaling system to use and engineer in engineered bacterial communication networks.

The AI-2 signaling system in V. harveyi and the C-6-HSL signaling system in P. aureofaciens are only two of the many currently identified quorum sensing systems identified. However, the vast majority of identified intraspecies and interspecies signaling systems function through similar principles and similar AHLs. Research indicates that many of these systems are controlled by a similar class of "R" and "I" proteins (PhzR type and PhzI type) throughout the bacterial kingdom [23]. Many interspecies signaling systems also surely remain yet undiscovered. Understanding the diversity of signaling mechanisms and variety of functions and purposes of these systems is paramount when working to engineer nature's natural sensing systems to use them as sensors, signals and other directed applications.

Background - Biological Information Processing Systems

One research group that has begun exploring engineered cellular communications with some success is Weiss et. al. They developed and tested a simple two-cell model that was capable of receiving, processing and sending information between engineered cells. The work, inspired by the bioluminescent quorum sensing system utilized by the marine bacterium Vibrio fisheri, uses the V. fisheri quorum sensing signal VAI messenger molecule as the informational unit [24]. The group modified E. coli DH5 alpha cells to produce the signaling molecule VAI when in the presence of anhydrotetracycline (aTc). They also developed a group of modified DH5 alpha receiver cells that bioluminesce in response to VAI.

To demonstrate the communication between cells, Weiss et. al. first created a cell that would emit a signal molecule based on a known input, aTc. The group accomplished this by controlling the expression of the luxI gene from V. fisheri, which is required for production of VAI, with a plasmid responsive to aTc. Thus, when aTc was present, the luxI gene was expressed and it resulted in the production and release of VAI, a small molecule, from the cell. Weiss modeled this cell in terms of the AND digital logic gate, where a constitutively expressed inactive activator is activated through an inducer molecule and then expresses the luxI gene. Weiss et. al. model the bacterial system after digital logic gates used in computer engineering.

Digital logic gates represent the combination of transistors, diodes and resistors found in traditional electronic wiring diagrams and on modern circuit boards. The specific combination of these components creates one of five (5) of the most common digital logic gates: AND, OR, XOR, NOR and NAND. Each of these gates output one of two signals, either 1 or 0, depending on the input values. Individual gates have specific arrays of conditions when the gate will output a 1 or 0. For example, an AND gate has two inputs and one output. If neither of the inputs is present, or either input is present alone, then the output is 0. Only when both inputs are present is the output of an AND gate 1. Weiss et. al. essentially built a biological AND gate that would respond to a single input (aTc) because they engineered the second input as a constitutively expressed element within the cell. To create the AND gate corollary in the cell, Weiss et al. incorporated one of the required components in the production of VAI on a constitutive plasmid. Modeling these biological systems as digital logic gates is an important aspect of these bacterial combinatorial networks, as it allows mathematicians and computer scientists to model the systems and predict system response and suggest starting parameters for concentration and time when conducting experiments.

Weiss et. al. also developed receiver cells made from E. coli DH5 alpha [24]. These cells contained the LuxRPLPR operon from V. fisheri that is expressed in the presence of VAI. Therefore, when VAI was present in the solution, the receiver cells would respond by expressing the LuxRP$_L$P$_R$ genes and green fluorescent protein (GFP). When

these receiver cells were coupled with the sender cells (VAI producing, aTc-responsive) and aTc was added, the receiver cells would produce GFP and luminesce[24]. In this way, Weiss et. al. created the first engineered cell to cell communication network loosely based on the theory of digital logic gates.

Another group, Thomas et al. from the University of California, Los Angeles (UCLA) also recently experimented with engineered cell to cell communication. Their experiments were based on manipulating the nitrogen regulation system and acetate pathway in E. coli to create an intercellular signal [25]. Thomas et al. also aims to utilize the power of quorum sensing between bacteria as a signaling system [26]. Acetate is used as the signaling molecule in these experiments, and the difference in pH across the membrane serves as the input signaling system. The receiver cells in this cell-cell communication network respond with GFP, the same output signal used in experiments by Weiss et al.. As was the case with the Weiss experiments, Thomas et al.'s work was directed largely by the prediction of a mathematical model. Through the model, the experiment clearly demonstrated that varying pH cause varying fluorescence output [26].

Background - Cyanobacteria

Cyanobacteria have the distinction of being some of the oldest known organisms, with fossils dating back nearly 3.5 billion years. Originally classified as blue-green algae, they are actually gram-negative bacteria in the archae classification. Cyanobacteria are found all over the world. Cyanobacteria have played an important part in evolution, particularly in plants, where the chloroplasts are thought to be descendants of cyanobacteria that took up host in the cells and eventually became part of the cell through endosymbiosis. These bacteria, while primitive in origin, have appealing characteristics for use in engineered cellular networks; cyanobacteria naturally respond to light, require minimal nutrition for survival and survive at a wide variety of environmental conditions from hot-springs to cold ocean water.

The light response of cyanobacteria is a useful characteristic to use in engineered systems [27]. Light can pose both a threat and benefit to cyanobacteria. On one hand, light is a source of energy for cyanobacteria, while too much light or high-energy wavelengths of light can be harmful. Research has identified specific photoreceptors that respond to particular wavelengths of light such as red or UV light [28]. Various strains of cyanobacteria, including cyanobacteria strains Synechococcus elongatus PCC 7942 [29] and Synechocystis PCC 6803 [30] have identified photoreceptors and light responsive genes.

From a synthetic biology viewpoint, cyanobacteria Synechococcus elongatus PCC 7942 is an excellent light responsive bacteria to evaluate, as researchers have not only identified the light responsive genes, but already cloned the promoters for these genes. Additionally, a blue/UV-A light, responsive gene system has been characterized [31]. The blue light response network is based on high light inducible proteins (HLIPs). HLIPs are proteins that are proposed to serve in a protective manner against potentially damaging light rays by aiding the cell in handling reactive oxygen species. In the experiments, blue or UV-A light was shown to stimulate transcription of the hliA gene [31]. Experiments demonstrated increased levels of transcription of the hliA gene in response to blue or UV-A light in times as short as 3 minutes [31]. Times shorter than 3 minutes were not investigated in the experimental models for response. Light induced signaling systems are targets for genetic engineering as they allow scientists to control engineered cellular activity via light, meaning that there is no actual contact between the engineered system and traditional electronic signaling equipment and no agents need to be added to the system. Previous studies have shown that some of light regulation in bacteria is post-transcriptional [32], but transcriptionally controlled light response is more useful for cloning and engineering cells.

Recently, the entire genome of cyanobacteria Synechocystis PCC 6803 has been sequenced. In fact, the genome for Synechocystis was the first cyanobacteria genome completely sequenced, making it the primary strain used for genetic studies in cyano-

bacteria as well as photosynthetic bacteria in general [33]. Similar to PCC 7942, strain 6803 has the ability to respond to light. Because the genome is sequenced in this strain, there has been ample research on incorporating foreign DNA into the chromosomal DNA of this strain as well as the production of a variety of plasmids that work well in cyanobacteria. Combined, the sequenced genome, the ability to survive at ambient temperatures and the ease of transformation make cyanobacteria synechocystis PCC 6803 an excellent choice as a foundation cell for engineered bacterial cellular networks.

Background - Clostridium perfringens

Clostridium perfringens is an anearobic, immobile, gram-positive spore-forming bacteria found naturally in the environment and the normal fecal flora of many individuals. There are five types of C. perfringens: Type A, B, C, D and E; the various types contain or express different toxins and are found in different environments. Few identified bacteria plague mankind in the variety of ways C. perfringens can. Part of the reason for the wide array of illnesses C. perfringens causes is the large number of toxins produced by the bacteria, each of which affects cells differently; the three most common and studied toxins are alpha toxin, kappa toxin and theta toxin. Some of the diseases caused by C. perfringens range from mild gastroenteritis from food poisoning to multiple organ failure, gas gangrene, necrotic enteritis, and antibiotic associated diarrhea. In fact, with 250,000 cases per year in the United States, C. perfringens is the third leading cause of foodbourne illness in our country [34]. There currently are few ways to rapidly identify the presence of C. perfringens in a wound or as the etiology in a food poisoning outbreak because, as an anaerobe, it is difficult and time consuming to culture and does not readily survive in air. Rather, most clinicians, especially in cases of potential gas gangrene, rely on clinical presentation, sound and smell to make a diagnosis. Depending on the disease, treatment options for C. perfringens are limited and often must be administered early after infection. Some of these treatments are unique and even include the use of hyperbaric oxygen therapy [35]. While C. perfringens is most often thought of as a threat to immuno-compromised people, its potential to inflict mortality, economic

hardship from medical costs and danger to armed forces personnel as food poisoning stands to potentially immobilize entire platoons of military personnel make C. perfringens a bacteria of interest to infectious disease researchers.

Gas gangrene, the most traumatic, visual and potentially fatal disease caused by C. perfringens, occurs when exposed wounds are infected with C. perfringens through unintentional exposure to environmental sources or even self-contamination through fecal and body samples. Gas gangrene infections have plagued humanity as far back as medical records exist, even though the etiology was not initially identified until 1892. They began to gain notoriety during the first World War, when large numbers of wounded soldiers succumbed to the fast acting disease [36]. Originally dubbed "gas edema," "mignant emphasema," "gas inflammation," and "gas phlegmon," gas gangrene was a major obstacle to World War I physicians because clinical diagnosis was difficult and unreliable and fulminant gas gangrene progressed rapidly [36]. Clinicians at the time who were able to feel gas pockets in the wound, tap the wound and hear a tympani-like percussion roll or see gas bubbling from an open wound were able to successfully diagnose a gas gangrene infection [36]; this detection method is still often employed today.

The gas gangrene infection itself manifests by myonecrosis, destruction of local muscle tissue, necrosis of other tissues in the area and shock [37]. Of all the identified C. perfringens toxins, alpha toxin (phospholipase C) and theta toxin (perfringolysin O) are

the two major toxins involved in the progression of gas gangrene [38]. Experimental evidence demonstrates that both phospholipase C (PLC) and perfringolysin O (PFO) are required for a gas gangrene infection to persists in the tissue [39]. PFO has been shown to perforate 20-30nm pores in cholesterol containing membranes [40], whereas PLC induces hemolysis via a series of different signaling cascades within the cell [41]. Unless treated very early with antibiotics, most often penicillin, or unless infected tissue is completed removed, gas gangrene can progress to mortality within 6 hours.

Fortunately, gas gangrene infections are relatively rare. Most cases occur in military field wounds, hiking accidents or climbing accidents, when the time to reach sufficient medical care and have wounds cleaned is long, thus allowing any C. perfringens spores that enter the open wound the opportunity to begin growing. Another common gas gangrene occurrence is in burn victims, where there is a lot of damaged and exposed tissue and generally a suppressed immune system. Sometimes, however, gas gangrene occurs in rare instances, even among healthy people with fatal consequences, further demonstrating the need for better detection mechanisms. One example is a case where a 57 year old live liver donor successfully underwent the donation without complication [42]. The following evening, the patient had a lobster dinner, and within 24 hours experienced nausea, altered mental capacity, tachycardia, which eventually led to cardiac arrest and death. The autopsy showed that a type of C. perfringens, Type D, which boasts another systemic toxin, epsilon toxin, cause gas gangrene in the stomach and eventually

led to mortality; the C. perfringens came from the lobster dinner [42]. Curing these types of C. perfringens infections is only effective at the very early stages and even then is only marginally effective. Undoubtedly, rapid detection is a must for a positive outcome.

The other major component of C. perfringens infections targets the intestines and results in varying levels and types of gastroenteritis and diarrhea. Often in cases of intestinal disease, it is difficult to determine if the infection initiated from something that was ingested or if it was a flare-up of C. perfringens in the microbial flora of the intestines, as 30% of the population are naturally colonized with C. perfringens [42]. While most of the time these infections are far less severe than gas gangrene, they comprise the vast majority of C. perfringens infections. These intestinal infections range from simple, mild gastroenteritis, to the common antibiotic associated diarrhea, necrotizing colitis and the rare disease, Pigbel. The target and action of C. perfringens varies in each type of infection. In antibiotic associated diarrhea, patients administered antibiotics have their natural intestinal microbial disrupted. C. perfringens then colonizes the intestines and the toxins cause diarrhea. The other intestinal infections vary in severity and specificity, with all types of people infected. One interesting case of an outbreak of C. perfringens at a mental facility following a Thanksgiving meal resulted in 2 deaths, of 7 cases of enterotoxigenic C. perfringens and 3 cases of acute necrotizing colitis [34]. The effects of foodborne outbreaks of C. perfringens and subsequent infections, while not always life

threatening, carry the economic burden of missed work hours and wages and associated medical costs for tracking the source and treating infected patients. The missed work hours pose an especially dire situation for military personnel.

Studies of C. perfringens and its associated disease states have been studied outside of humans as well. As an example, an outbreak of necrotizing enteritis linked to C. perfringens plauqed a Korean ostritch farm in 2000 and brought significant economic hardship on the farmers because of the loss of livestock [43]. Other research groups have evaluated C. perfringens growth and persistence in food. For example, one study by the Dutch Inspectorate for Health Protection and Veterinary Public Health in 2001 demonstrated the presence of C. perfringens in canned soup [44]. Additional rapid testing techniques to identify C. perfringens in food prior to consumption or distribution could result in decreased infections and a better economic outlook for farmers, food packagers and potentially ruined restaurant chains over poor publicity following inadvertent outbreaks.

B-Mad System

Introduction to B-Mad System

The Bacterial-based Molecular Assay Detection System [B-MAD System] for C. perfringens is designed for both military and civilian applications. It is an engineered network of bacterial cells designed to detect C. perfringens. The portability, compact size, minimal nutritional requirements and rapid response of the B-MAD system is ideal for the military. The system will allow military personnel aboard vessels and forward bases to test foodstuffs for potential food poisoning, identify C. perfringens infections quickly in combat wounds, even during transportation to the nearest trauma unit, and track and contain the sources of C. perfringens food poisoning outbreaks. Additionally, the B-MAD System is a useful diagnostic tool for clinicians at hospitals and rural medical clinics. It provides an epidemiological tool for researchers at public health departments tracking and identify food poisoning etiologies. Farm operators could also look to the B-MAD system as a method to contain C. perfringens outbreaks in swine, ostrich or other livestock.

Harnessing cyanobacteria's minimal nutritional requirements, self-reproducibility and natural capability to respond to environmental stimuli and communicate, it is possible to develop new, modular, detection systems based solely on engineered bacteria for detecting infectious diseases, toxins and small molecules. The natural sensitivity and specificity of biomolecular systems, combined with intercellular commu-

nication among bacteria, allows for detection of target molecules; a unique biologically-based reporter system provides a user interface to the system. An initial application of the Bacterial-based Molecular Assay Detection System [B-MAD System], chosen as a proof of concept, is the detection of C. perfringens in wound swabs, fecal samples, and food.

The B-MAD System uses bioluminescence and biofluorescence as outputs. The genetic elements making up the B-MAD System are cloned from naturally occurring bacteria communication and sensing systems. Cyanobacteria make up the backbone of the B-MAD System, and since cyanobacteria readily survive at room temperature for extended periods of time, the B-MAD System will be extremely portable. The B-MAD System is flexible and designed to have interchangeable and customizable components to serve as a detection or relay system for numerous toxins or bacteria. Each application of the B-MAD System detects extra-cellular molecules specific to the target bacteria.

B-MAD System Design

The B-MAD sensing system is a modular, completely biological device consisting of a network of engineered bacteria containing an "on/off" switching module and two combined "sensing" and "processing" modules. The B-MAD system responds to three separate inputs: 1) blue/UV-A light as a "start-up" signal, 2) perfringolysin O (PFO), a C. perfringens toxin, and 3) AI-2, an extra-cellular molecule produced by C. perfringens. When combined, these three inputs result in the detection of C. perfringens in a sample (see Figure 1).

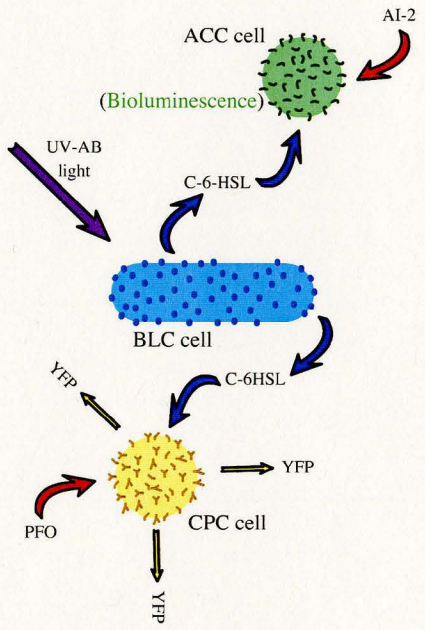


Figure 1: Illustration of B-MAD System response to stimuli (UV light & signaling molecules).

The B-MAD system has three bacteria cell types that serve as the "processor" of the system. Cyanobacteria were chosen as processing cells, as they grow, live and remain alive for extended periods of time without specific maintenance at ambient room temperature. As well as being robust, cyanobacteria naturally respond to various wavelengths of light in different ways, an important characteristic for the system because the molecular systems necessary for light responsiveness are intrinsic to the bacte-

ria. Cyanobacteria strains 7942 and 6803 serve as host cells; genetic engineering of these strains will create the B-MAD system. Strain PCC 7942 is the host cell for the blue light converter or system activator. The other strain, 6803, serves as the host cell for the two combinatorial processor cells. The system itself is divided into three separate cell components: a Blue Light Converter cell type (BLC), an AI-2/C-6-HSL Computational cell type (ACC) and a C-6-HSŁPFO Computational cell type (CPC). These three cell types, combined in a modular fashion, provide the detection of C. perfringens presence.

Prior to use, the B-MAD system is inactive, contained in a microcentrifuge tube, cuvette or other small container useable in portable fluorimeters and luminometers (see Figure 2). The blue/UV-A light activates the system through the Blue Light Converter cell prior to exposure to potentially infected fluids, tissue swabs or fecal material. Upon activation, the system will fluoresce yellow, which serves as an "on" indicator. The positive signal indicates to users that the system is both active and working; it also provides a baseline system fluorescence level. After activation, the system is exposed to the potentially infected material. If there is AI-2 present, the system will bioluminescence (produce a blue-green light) via the ACC cell, which detects C-6-HSL and AI-2. If there is PFO present, there will be a change in yellow fluorescence (either a distinct dimming or increase

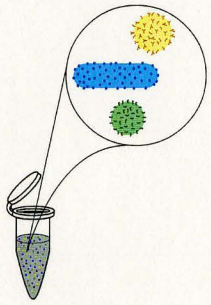


Figure 2: B_MAD system sketch illustrating three cell types in potential system packaging.

in brightness, depending on system preliminary test results) from the CPC cell, which detects C-6-HSL and PFO. A change in yellow fluorescence, combined with bioluminescence, indicate C. perfringens presence. Bioluminescence and a change in yellow fluorescence provide an unambiguous system output.

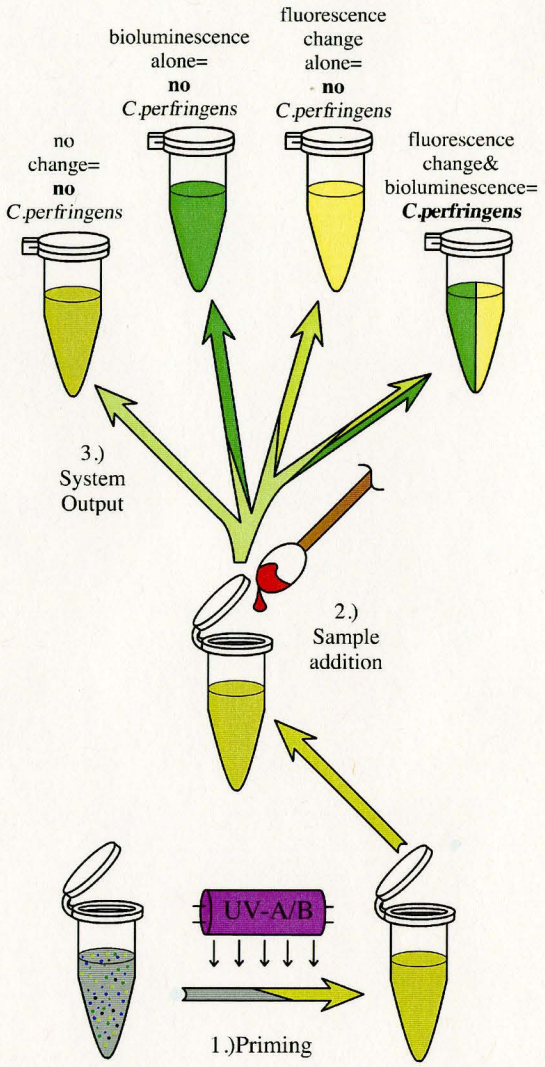


Figure 3: Illustration of B-MAD system in use and possible system outputs.

Each of the two processor cell types (ACC cell and CPC cell) detects the activation signal, blue/UV-A light, and one of the signaling molecules. Thus, the two processor cells, together, determine if there is C. perfringens in the sample when both are activated and each senses one of the signaling molecules (i.e.: respond indicating AI-2/C-6-HSL positive and PFØ C-6-HSL positive. See Figure 3). The B-MAD system is able to use the combination of inputs to separate the detection of C. perfringens from other bacterial, fungal, toxin or

viral infections, as PFO and AI-2 production together is unique to C. perfringens. Additionally, other bacteria that may produce AI-2, PFO or similar molecules to PFO or AI-2 would not inhabit the same environment. Therefore, the B-MAD system is able to detect C. perfringens relying solely on bacterial sensing mechanisms.

The B-MAD system is an analogue to digital logic AND gates, which are digital signal processing units that receive two inputs and

AND Gate "1" from figure

C-6-HSL	AI-2	Biolum.
no	no	no
yes	no	no
no	yes	no
yes	yes	yes

AND Gate "2" from figure

C-6-HSL	PFO	Δ in fluor.
no	no	no
yes	no	no
no	yes	no
yes	yes	yes

AND Gate "3" from figure

Biolum.	Δ in fluor.	C. perf.
no	no	no
yes	no	no
no	yes	no
yes	yes	yes

provide a single output; if both inputs are positive, the AND gate signals a 1, or positive output, while in all other cases it signals a zero, or negative output. As shown in the figure on the right, both the ACC and CPC processing cells are analogous to digital logic AND gates. Additionally, the change in yellow fluorescence and bioluminescence work together as a digital logic AND gate. Globally, the entire B-MAD system can be viewed as a digital logic AND gate, with PFO and AI-2 serving as the inputs giving a positive or negative output determining whether or not there was C. perfringens present. The following tables correspond to

Digital Signal Processing AND Gate

Input 1	Input 2	Output
0	0	0
1	0	0
0	1	0
1	1	1

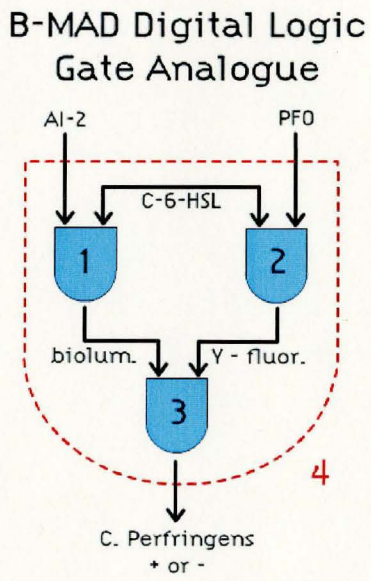


Figure 4: B-MAD Analogue

the digital logic AND gates in the schematic. These tables demonstrate the relationship between the AND gate used in digital signal processing and the AND gate analogues in the B-MAD system.

AND Gate "4" from figure: Global B-MAD AND Gate

AI-2	PFO	C. perf.
no	no	no
yes	no	no
no	yes	no
yes	yes	yes

Blue Light Converter Cells (BLC)

The following section is a design and construction plan for the Blue Light Converter cell. The section includes a detailed design of the BLC cell, including all required components and their role in creating the BLC cell. An experimental construction plan is included laying out the steps and specific cloning required to construct the BLC.

The Blue Light Converter, engineered cyanobacteria PCC 7942, is the B-MAD system activator, providing visual feedback that the system is viable and functioning while also prepping the other processing cells for detection. In response to UV-A/B light, the BLC cells produce and secrete C-6-HSL, a small intercellular signaling molecule normally found in Pseudomonas aureofaciens. This molecule serves as the activating signal for both computational cells.

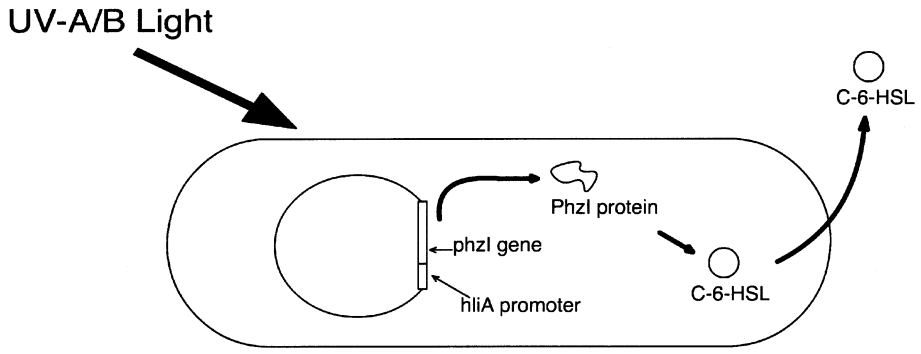


Figure 4: Illustration of the Blue Light Converter cell (engineered cyanobacteria 7942), components and response to Blue or UV light.

Cyanobacteria strain PCC 7942 reacts to UV-A/B light by upregulating the hliA genes. In nature, these genes produce molecules that protect the bacteria from the harm-

ful effect UV light has on cellular components. Coupling the hliA promoter to the phzI gene on a plasmid within cyanobacteria PCC 7942 creates the Blue Light Converter. The BLC harnesses this intrinsic response to UV light stimulation by utilizing the hliA promoter to induce transcription of the gene coding for the precursor substrate to the C-6-HSL molecule, phzI, in response to UV light. Using this newly engineered cell, it will be possible to create C-6-HSL in response to UV light. The C-6-HSL molecule interacts with the the 1st computationałdetector cell (CPC), causing transcription and translation of yellow fluorescent protein (YFP) via C-6-HSL dependent YFP transcription. In the second computationałdetector cell (ACC), C-6-HSL induces the transcription and translation of luxP, the AI-2 receptor.

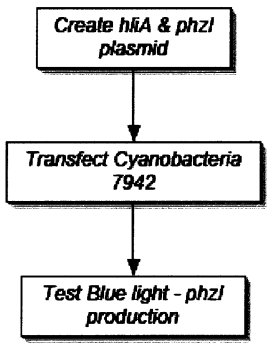


Figure 5: Construction flowchart for BLC cell.

A staged construction is planned to construct the Blue Light Converter, consisting of two major steps: cloning and transformation. To create the BLC, the hliA promoter will be cloned into a plasmid for cyanobacteria. Then, cloning the phzI gene into the cyanobacteria plasmid adjacent to the hliA promoter creates the final plasmid. After transformation of the cyanobacteria, exposure to Blue or UV-A light and successful assay to determine C-6-HSL production, the BLC is complete.

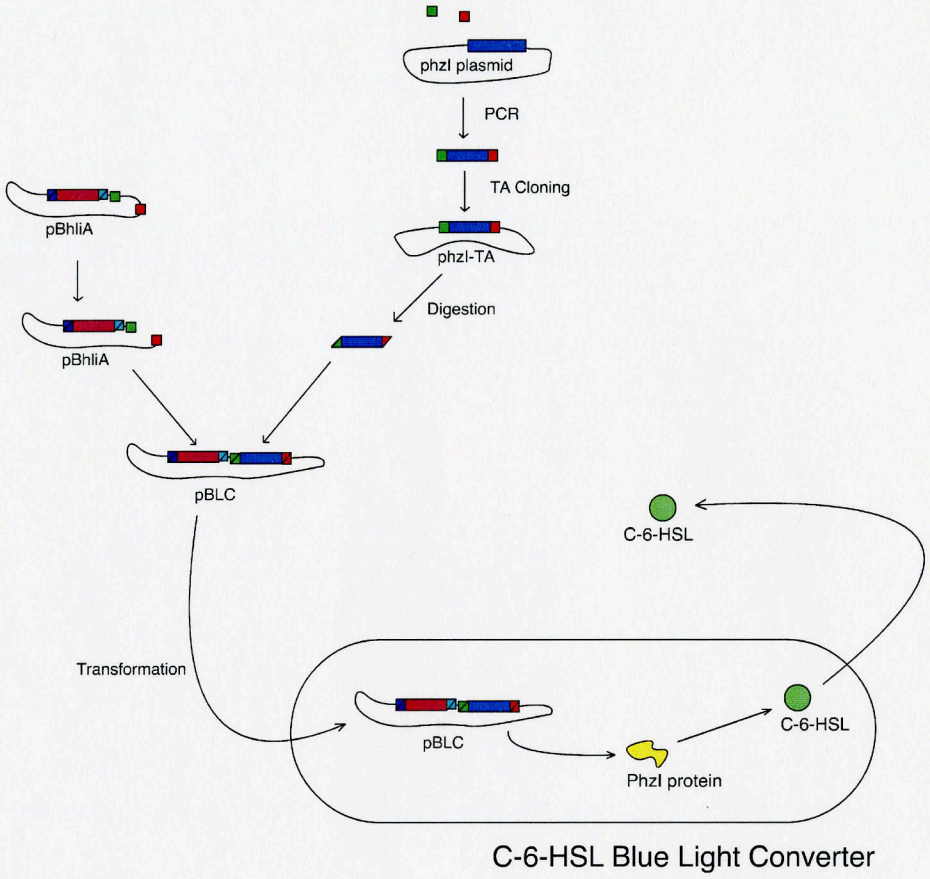


Figure 6: Construction outline for the BLC converter cell, including all intermediate plasmids required for construction of the BLC.

C-6-HSŁPFO Computational Cell (CPC)

The following section is a design and construction plan for the C-6-HSŁPFO Computational (CPC) cell. The section includes a detailed design of the CPC cell, including all required components and their role in creating the CPC cell. An experimental construction plan is included laying out the steps and specific cloning required to construct the CPC cell.

The C-6-HSŁPFO Computational cell (CPC) demonstrates system activation with the production of yellow fluorescent protein (YFP), which is also required for detection of the C. perfringens toxin perfringolysin O (PFO). PFO interacts with cholesterol in the plasma membrane of eukaryotic cells to form pores, which causes the cellular contents to spill out into the extra-cellular space, killing the cells. PFO will be detected through a similar mechanism in the B-MAD system. In the presence of PFO, CPCs will form pores and die, resulting in YFP diffusing into the extra-cellular space, affecting the fluorescence signal. There are two potential fluorescent signals visualized once the YFP escapes the cells. In either case, the change in fluorescence registered by the system will count as a PFO presence. When the YFP escapes the cells through pores formed by PFO, either the YFP intensity registered will decrease because it is now less concentrated within the cell or the overall YFP signal may increase because of interactions with extra-cellular molecules present. Testing of the CPC cell will allow for calibration in either case. With the production of YFP and interaction with PFO, the CPC cell serves not only as a detec-

tor of PFO, but also as a bioassay for PFO activity.

In nature, Pseudomonas aureofaciens, a plant pathogen, employs a quorum sensing system to detect bacterial numbers. This quorum sensing sensing detection system responds to the presence of a small molecule, C-6-HSL. This small molecule interacts with PhzR, a transcriptional activator for the phzE promoter (see Figure 7, #1).

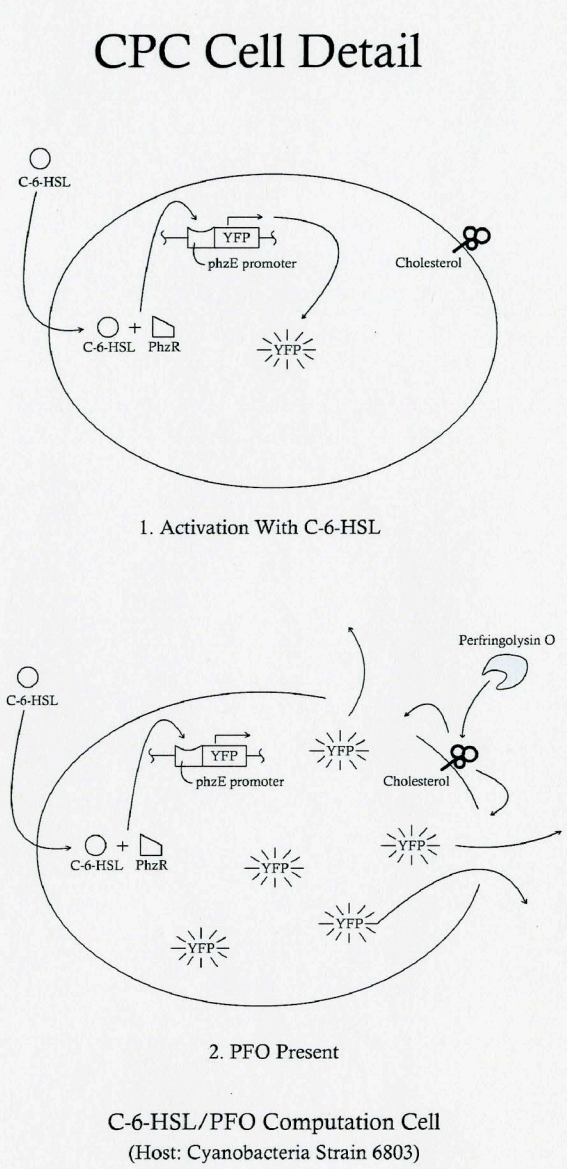


Figure 7: Illustration of CPC Cell and mechanism of action

Using the C-6-HSL response network from P. aureofaciens, the PhzR protein and its target gene promoter phzE, YFP production occurs when C-6-HSL is present in the extra-cellular fluid. The C-6-HSL molecules diffuse across both cell membranes, bind to the PhzR protein in the cytosol and directly promote the transcription of the gene attached to the phzE promoter, in this

case YFP (in the engineered construct). In the B-MAD, C-6-HSL is derived from the BLC. Thus, light activation of the BLC leads to C-6-HSL production, leading to "activation" of the CPC cell, causing it to fluoresce yellow (see Figure 7, #2). These components will be added to the chromosome of the cyanobacteria 6803 host. Addition of DNA to the chromosome in the cyanobacteria is simple, which is one of the reasons it was chosen as a host. Chromosomal DNA is more stable than plasmid DNA, which should help to stabilize the entire B-MAD system.

In eukaryotes, PFO binds cholesterol in the cellular membrane and forms large pores, exposing the cytoplasm to the environment and resulting in cell death. Cyanobacteria 6803 do not normally contain cholesterol in its membranes. Therefore, we will have to engineer them to contain cholesterol. Some prokaryotes, particularly obligate intracellular pathogens like Ehrlicia and Chlamydia, incorporate cholesterol from the host cell into their membranes to provide additional support and stability for the cell wall. The concept is to stimulate the cyanobacteria cells to incorporate the cholesterol into the membrane. A second solution, if there is difficulty stimulating the cyanobacteria to incorporate cholesterol into the membrane, involves adding the necessary processing genes required to form cholesterol naturally in the cyanobacteria host cell, strain 6803. A third option includes the potential use of ergosterol, a structurally similar molecule to cholesterol found in parasites; the PFO may also work against ergosterol. Once the cholesterol is added, perfringolysin O toxin will be directly added to the solution to deter-

mine the effects on the modified cyanobacteria cells; these tests will indicate time required for toxin action and amount of toxin required to elicit the desired response.

To develop the CPC cell, the C-6-HSL receptor proteins and responsive genetic units, including the YFP gene, must be incorporated into the cell, which must then have its membrane cholesterolized (see Figure 8). Cholesterolizing the membrane and developing an C-6-HSL strain can be completed in parallel. Focusing on the addition of cholesterol to the membrane, there is a chance that the cyanobacteria cell membranes will naturally take up cholesterol into their membrane. Other potential solutions involve incorporating ergosterol into the membrane or cloning the genetic machinery required to allow the cyanobacterial 6803 to produce cholesterol for the membrane. The hope is that experimentation can show cyanobacteria 6803 will uptake cholesterol without significant engineering.

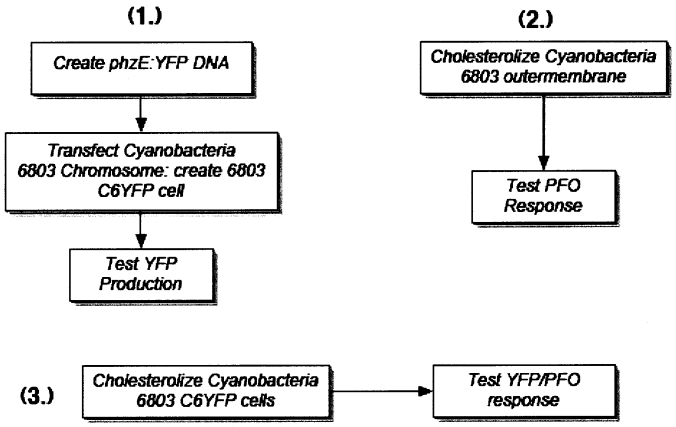


Figure 8: Task list and flow chart for construction of CPC cell.

Meanwhile, cloning approach will produce the phzE:YFP cyanobacteria DNA and constitutively expressed DNA for phzR, the C-6-HSL receptor. PhzR protein, since it is required for the reception of and successful response to C-6-HSL, must be present in

the cell. To achieve this, a constitutive cyanobacteria promoter can be linked to the phzR gene, ensuring transcription and expression of the phzR gene in cyanobacteria and, thus, the phzR protein. The promoter for the phzE gene, target of PhzR and controlling the YFP expression, will be linked via restriction enzymes to the YFP gene. Because of the nature of cyanobacteria 6803, the cyanobacteria will be transfected (adding the DNA to the chromosome instead of transforming with a plasmid) so that the phzE promoter: YFP construct and the constitutive promoter: phzR gene integrate into the cyanobacteria chromosome, providing a more stable engineered cell.

The created strain of cyanobacteria 6803 containing the CPC genetic elements can then be treated and engineered as required to incorporate cholesterol through methods explored on wild-type cyanobacteria 6803. See Figure 9, 10 and 11 for detailed construction diagrams.

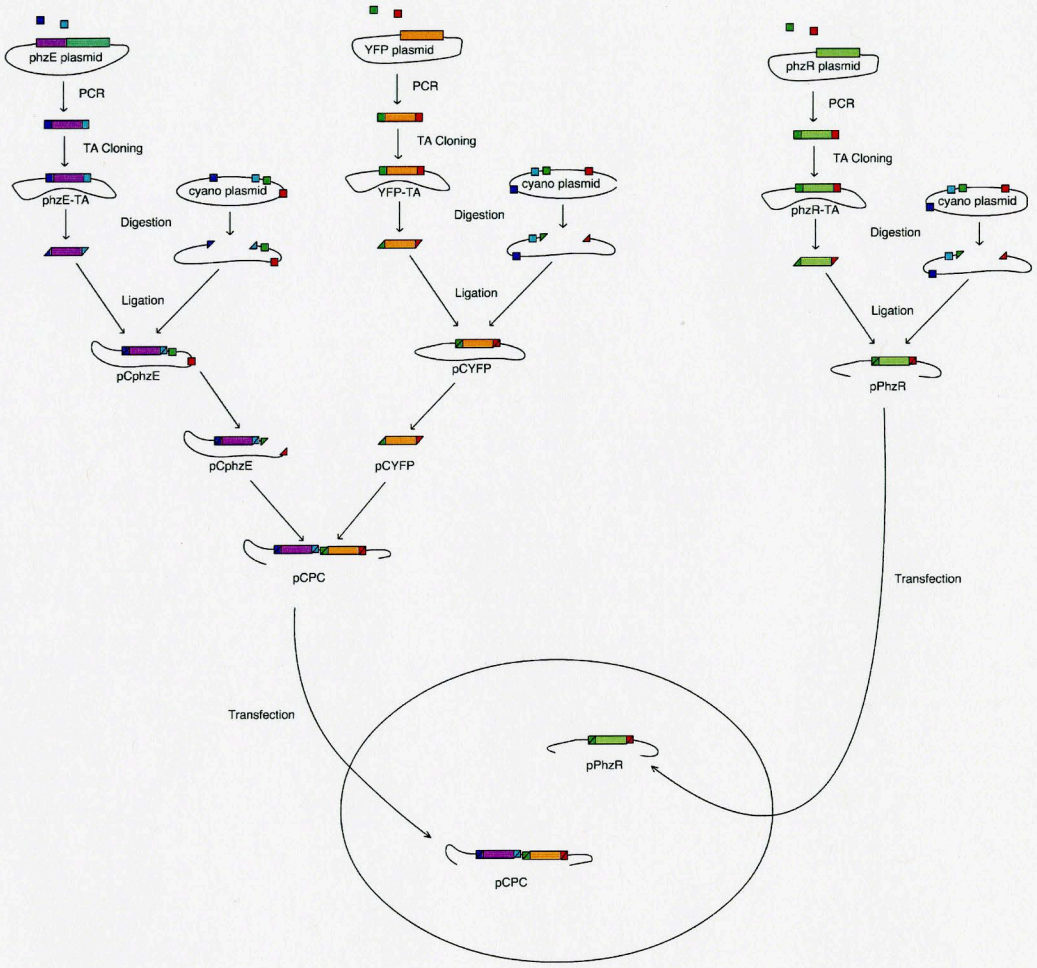


Figure 9: Construction outline for adding C-6-HSL responsive components in the CPC cell.

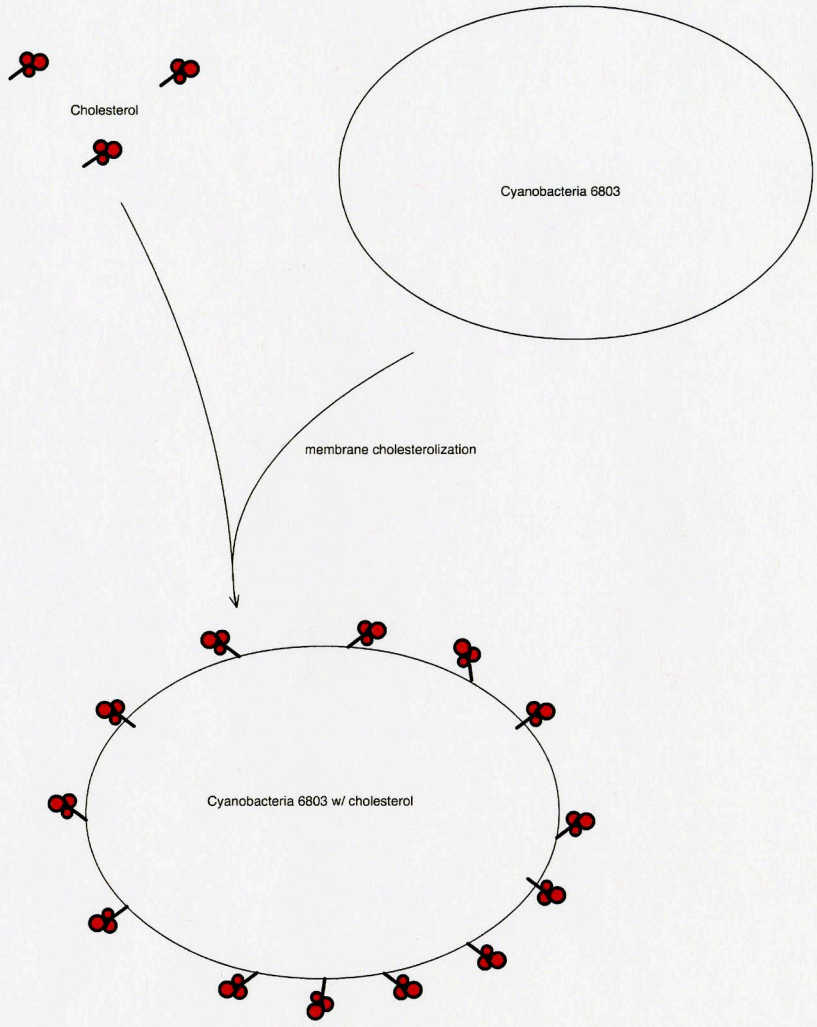


Figure 10: Construction outline for PFO toxin response in the CPC cell: addition of cholesterol to cyanobacteria 6803 membrane

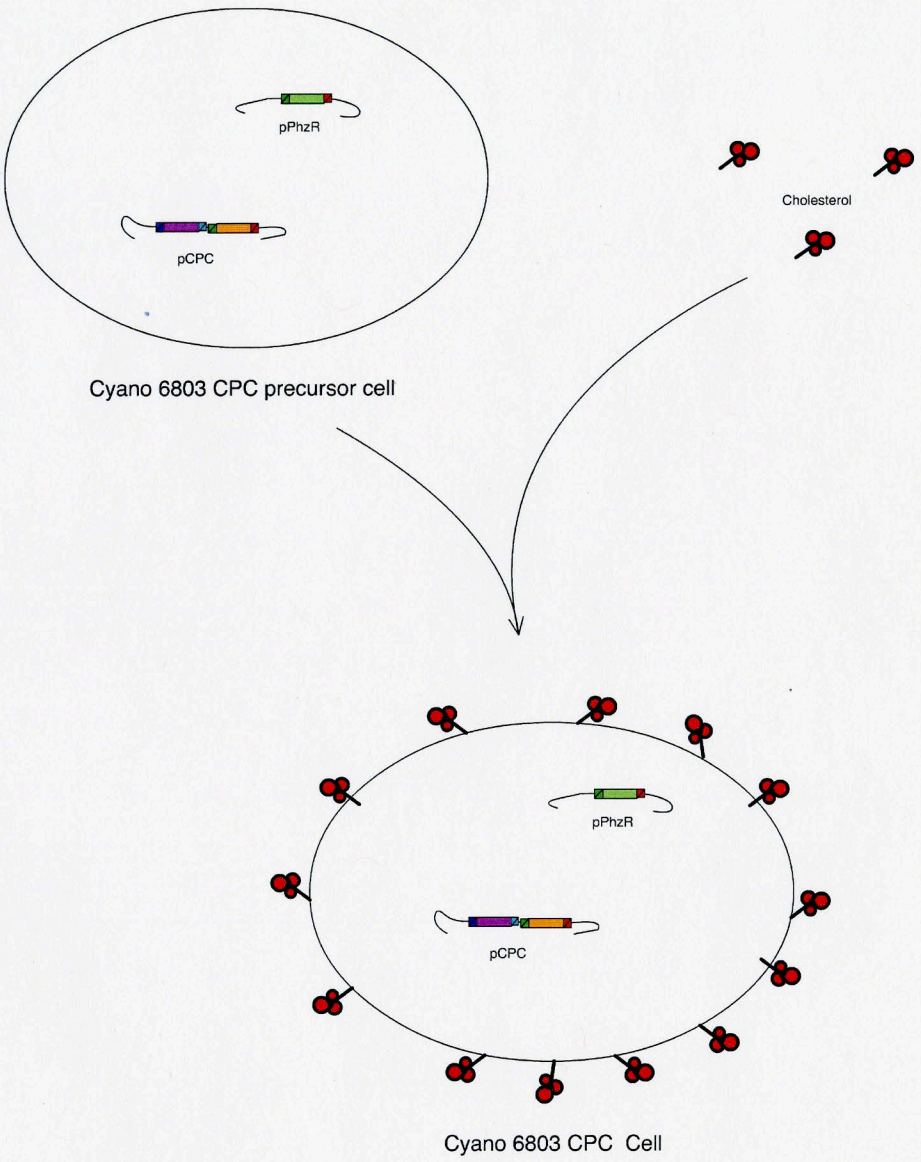


Figure 11: Construction outline for completion of CPC cell. The C-6-HSL responsive cell is cholesterolized to form the CPC cell.

AI-2/C-6-HSL Computational Cells (ACC Cells)

The following section is a design and construction plan for the AI-2/C-6-HSL Computational (ACC) cell. The section includes a detailed design of the ACC cell, including all required components and their role in creating the ACC cell. An experimental construction plan is included laying out the steps and specific cloning required to construct the ACC cell.

The AI-2/C-6-HSL Computational (ACC) cell detects the presence of AI-2, a small, non-toxic molecular product of C. perfringens. Initially described in the marine bacterium, Vibrio harveyi, AI-2 can be found in over 30 different bacteria species from E. coli to V. cholera (but not cyanobacteria). The identification of AI-2 alone in the media does not definitively indicate a C. perfringens infection, but when coupled with the CPC cell that senses the C. perfringens toxin perfringolysin O, a definitive diagnosis is possible; no other known bacteria produces this combination of products.

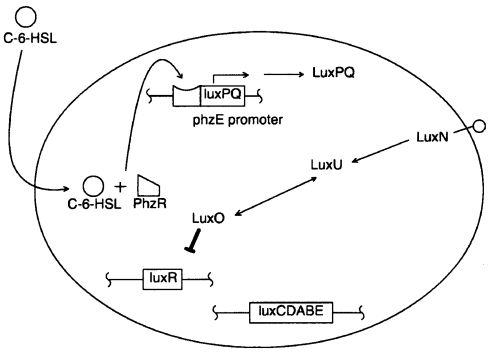
The ACC cell is "activated" by the C-6-HSL produced by the BLC cells following priming with UV-A/B light. When C-6-HSL is present, the ACC cell produces the receptor for AI-2, now making the ACC cell responsive to AI-2 and able to bioluminesce. Without the AI-2 receptor, the ACC cell is unresponsive to AI-2 in the media; thus, with AI-2 or C-6-HSL alone, there would be no bioluminescence; bioluminescence is only possible when both C-6-HSL and AI-2 are present or when AI-2 is present and the cells have already been primed with C-6-HSL.. To achieve this goal, biochemical sensing sys-

tems from Pseudomonas aureofaciens and Vibrio harveyi are introduced into cyanobacteria strain 6803.

As discussed for the CPC cell, Pseudomonas aureofaciens, a plant pathogen, employs a quorum sensing system to detect bacteria numbers. This quorum sensing system detection system responds to the presence of a small molecule, C-6-HSL. This small molecule interacts with PhzR, a transcriptional activator for the PhzE promoter. In P. aureofaciens, the phzE gene codes for the phenazine biosynthesis protein.

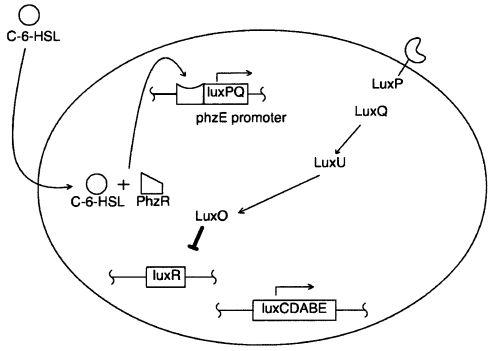
The marine bacteria Vibrio harveyi possesses a well-documented AI-2 sensing system. The backbone of the AI-2 detection system consists of four separate proteins: LuxP, LuxU, LuxO and LuxR. LuxP is the trans-membrane receptor for AI-2. In the AI-2 signaling pathway, AI-2 in the extracellular fluid crosses the outer cell membrane of the gram-negative V. harveyi and binds to the LuxP protein stretching into the periplasm outside the inner cell membrane. LuxQ now swtiches from a kinase to a phosphatase [11], and the phosphate is removed from LuxO; LuxO is inactive when unphosphorylated and cannot promote transcription of small regulatory RNA's (sRNA) that destabi-

lize luxR mRNA [2]. Thus, LuxR is translated and induces bioluminescence by promoting luxCDABE transcription, the bioluminescence genes, allowing V. harveyi to naturally bioluminesce in response to AI-2.

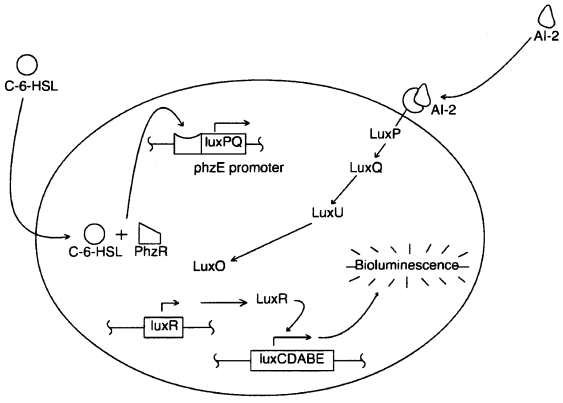


1. Activation With C-6-HSL

The ACC cells take advantage of both the Pseudomonas and Vibrio sensing pathways to detect AI-2. In the engineered ACC cyanbacteria cell (see Figure 12), C-6-HSL diffuses through the outer membranes where it interacts with PhzR, a P. aureofaciens protein. C-6-HSL binds to PhzR, a transcription factor, leading to the transcription of the luxP gene via the phzE promoter, a phzR responsive element. The luxP genes driven by the pzE promoter code for the luxP protein, the "reporter" in the *V. harveyi* AI-2 signaling pathway. Without LuxP, the ACC cell in incapable of bioluminescence in



2. No AI-2 Present



3. AI-2 Present

Figure 12: Illustration of AI-2 layout and mechanism of action.

response to AI-2. Additionally, LuxQ provides the necessary autophosphorylation of LuxO to ensure that expression of luxR is inhibited unless AI-2 is present and bound to LuxP.

There are a multitude of genes and promoters required to complete the ACC cell, and a clear construction plan is required to ensure all elements are properly added (see Figure 13). To simplify the construction of the ACC cell, the two receptor pathways and response systems, C-6-HSL and AI-2, will initially be cloned into separate cells to verify each system independently prior to combination of both systems in the final ACC cyanobacteria 6803 cell.

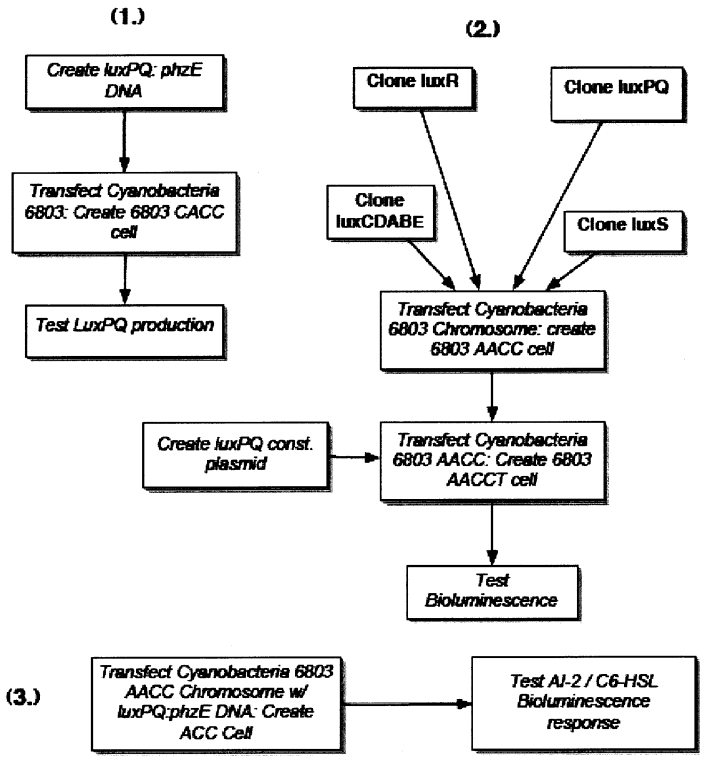


Figure 13: Construction flowchart for ACC cell. The flowchart illustrates a design mechanism for the ACC cell.

In the C-6-HSL response component ACC precursor cell, DNA coding for the luxP gene must first be linked to the phzE promoter. Additionally, the phzR gene must be linked to a cyanobacteria constitutive promoter. Then, both of these elements, inde-

pendently, may be added to cyanobacteria 6803 chromosomal DNA via transfection. Using this stable test cell, it is possible to tag the luxP protein with a 6 histidine tag to be used as a verification of response to C-6-HSL assay.

In the AI-2 response component ACC precursor cell, multiple DNA components must be cloned, linked and transfected into the cyanobacteria 6803 chromosomal DNA. The luxCDABE operon from V. harveyi and associated promoter region must be cloned and then added to the cyanobacteria. Similarly, the luxR gene must be cloned and incorporated into the cyanobacteria. Next, luxQ, luxU and luxO must all be linked to a cyanobacteria constitutive promoter and then added to the cyanobacteria chromosomal DNA. After verifying the successful addition of each of these components, preferably through sequence analysis, the ACC precursor cell must be tested for AI-2 response viability. However, the AI-2 receptor, LuxP, is not incorporated into the cell. As a testing mechanism, the luxP gene will be linked to a constitutive promoter on a cyanobacteria plasmid. The AI-2 response component ACC precursor cell will then be transformed with the luxP constitutive plasmid. Then, supernatant from the BLC_AI initially constructed and exposed to blue or UV-A light will be added to the ACC precursor cell to identify its ability to respond via bioluminescence.

After verification that both the AI-2 and C-6-HSL response systems are viable in the respective ACC precursor cells. The linked DNA for the C-6-HSL response component ACC precursor cell, luxP:phzE promoter and pzhR: constitutive promoter, will be

incorporated in the chromosomal DNA of the AI-2 response component ACC precursor cell, creating the final ACC cell. The ACC cell can then be exposed to C-6-HSL and AI-2 to test its overall response. See Figures 14 to 17 for detailed construction diagrams.

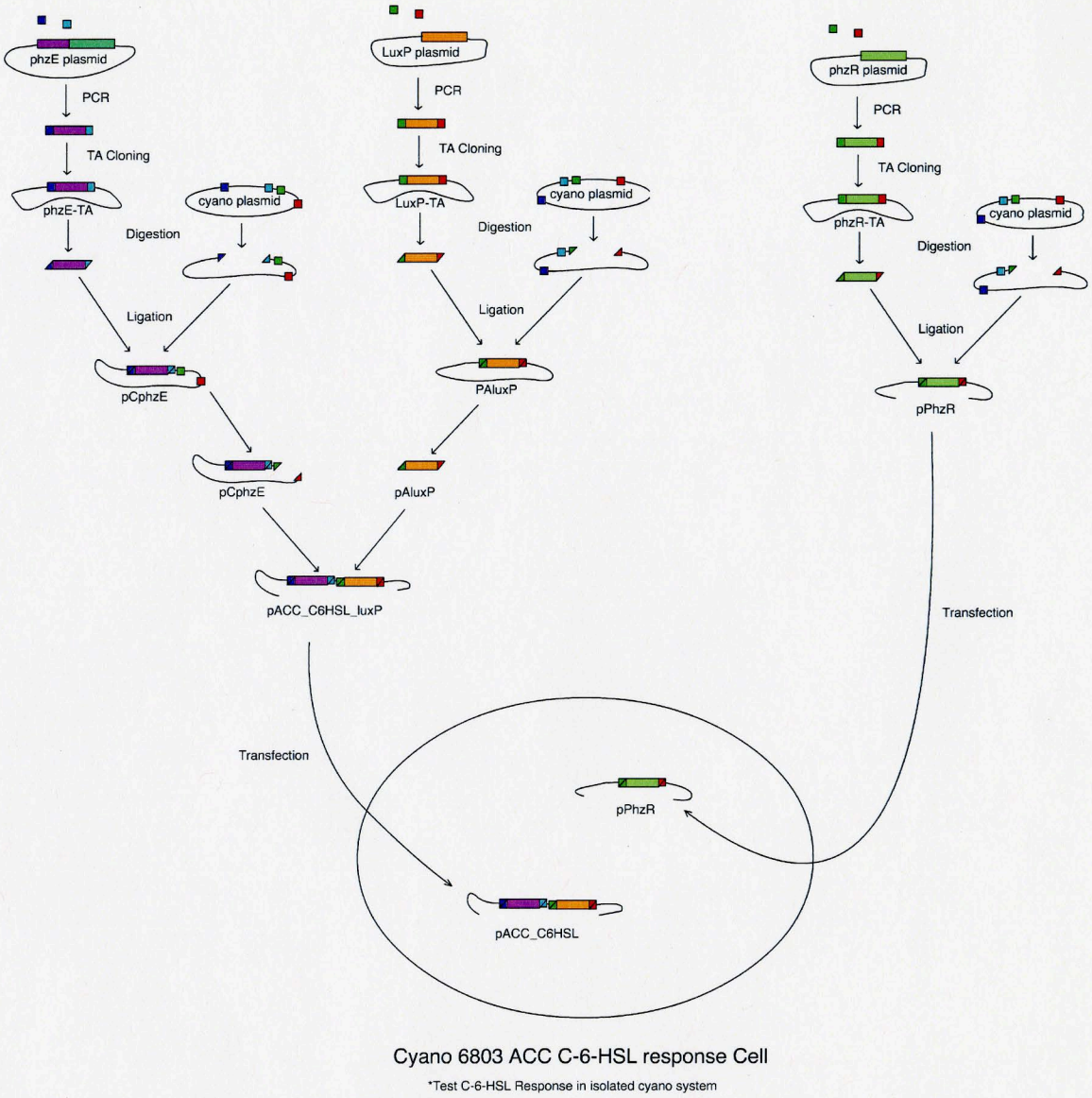


Figure 14: Construction outline for a C-6-HSL responsive system for the ACC cell that will produce the luxP AI-2 receptor protein.

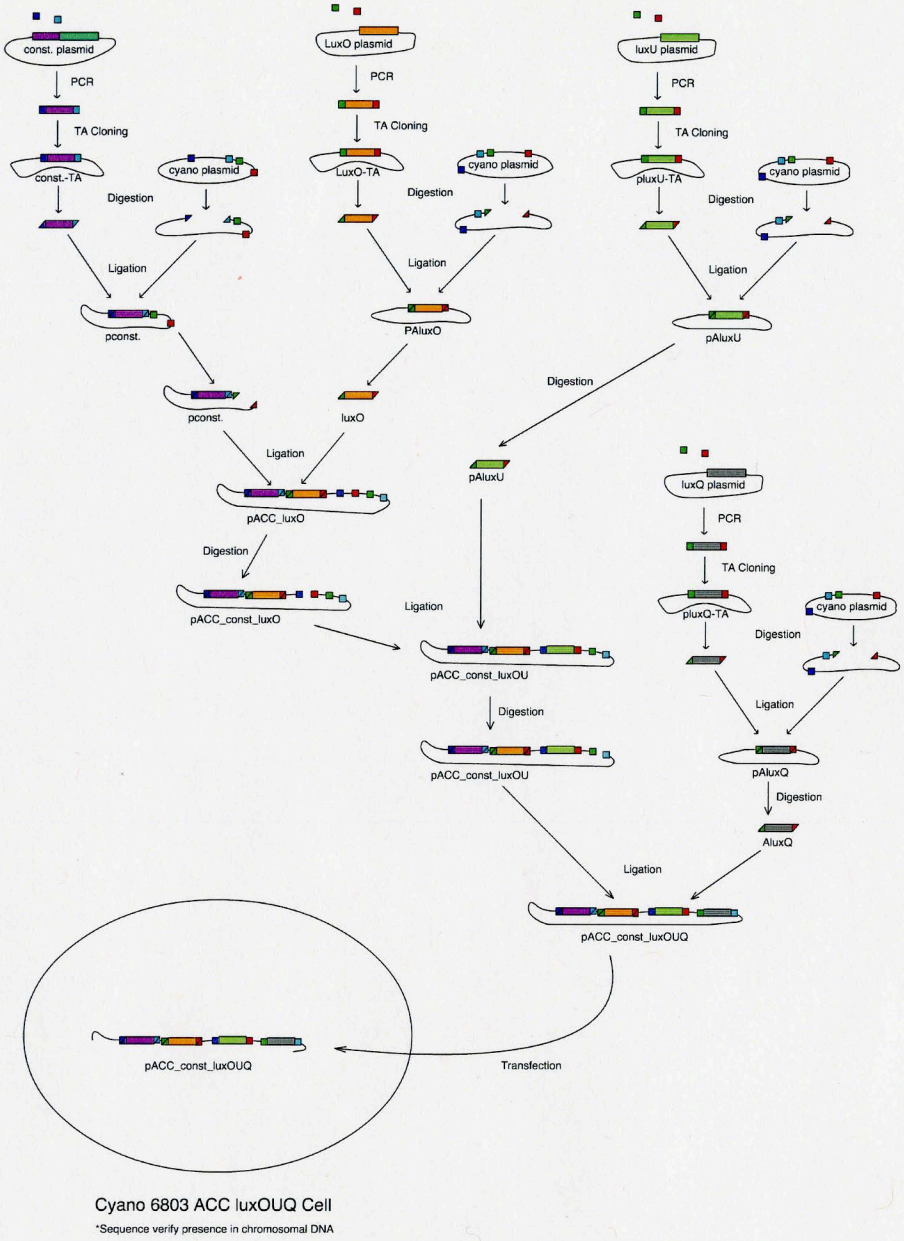


Figure 15: Construction outline for constitutive components required for construction of the ACC cell.

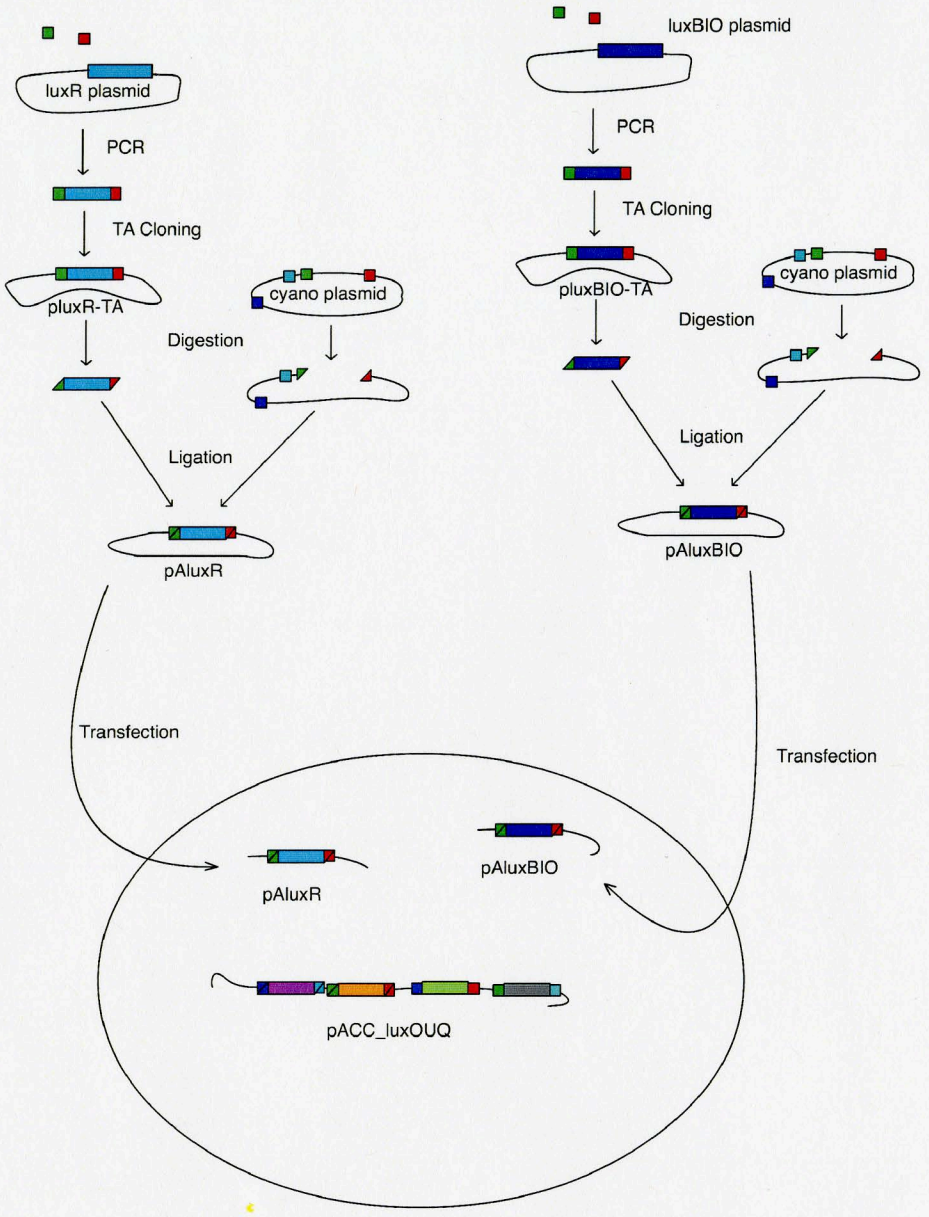


Figure 16: Construction outline illustrating addition of AI-2 responsive elements and the luxR gene and promoter sequence to the ACC luxOUQ cyanobacteria cell, creating the ACC AI-2 response precursor cell ACC.

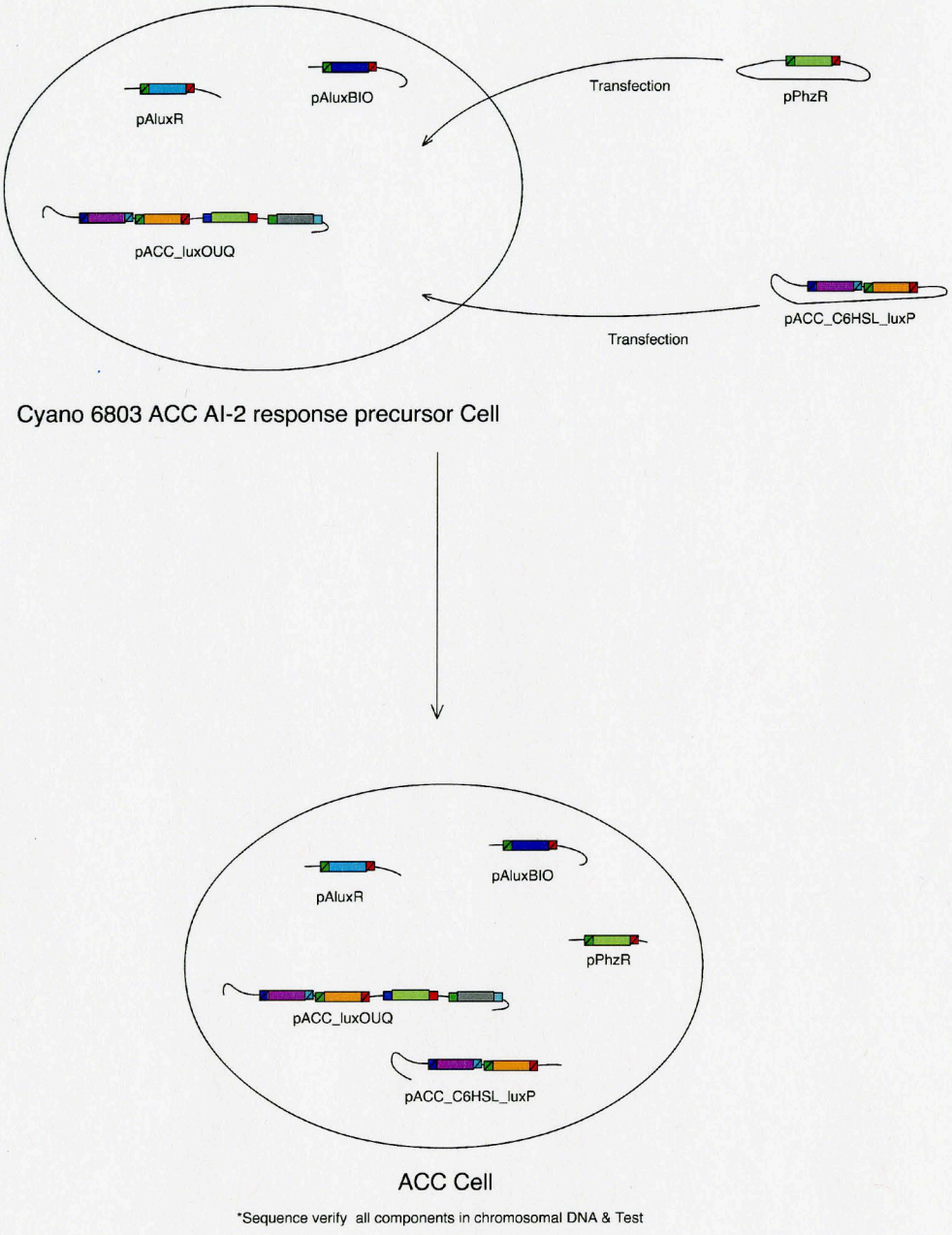


Figure 17: Construction outline illustrating addition of C-6-HSL responsive elements for luxP production for C-6-HSL ACC precursor to the ACC AI-2 responsive precursor cell to create the final ACC cell.

Blue Light Converter Component Construction

Experimental Plan

As a proof of concept demonstrating the physical potential to experimentally create the Bacteria-based Molecular Assay Detection [B-MAD] system that detects Clostridium perfringens, construction began on the initial Blue Light Converter (BLC_AI) cell and its components. While cloning and construction of plasmid DNA was taking place, work was also underway on growing and sustaining cyanobacteria strain 6803, cyanobacteria 7942 and a reporter strain of V. harveyi for use in the development and experimentation of the B-MAD system. Overall, three main experimental tasks were undertaken in this proof of concept work: cyanobacteria growth, V. harveyi growth and cloning of genes required to construct the Blue Light Converter (AI-2 version) cell.

Cyanobacteria Growth

At about the same time, we received plates of two separate strains of cyanobacteria, cyanobacteria synechocystis 6803 from Wim Vermaas at Arizona State University and cyanobacteria synechococcus elongatus PCC 7942 from Lorraine van Waasbergen at the University of Texas at Arlington. Both strains of bacteria were grown in a BG-11 media recipe provided by Vermaas that was altered to allow for the use of commercial BG-11 broth (see Figure 18). In the liquid and agar cultures of both cyanobacteria 6803 and 7942, the final glucose concentration, when used, was 5mM. While both cyano 7942 and cyano 6803 auto-fluoresce red, each of the two strains are morphologically distinct.

BG-11 Liquid Media (1L)	
1636.7 mg	BG-11 Broth (Fluka)
38.9 mg	MgSO4 (7 H20)
1 ml	Trace Minerals

BG-11 Agar Plates (1L)	
1636.7 mg	BG-11 Broth (Fluka)
38.9 mg	MgSO4 (7 H20)
1 ml	Trace Minerals
10 ml	1 M TES/NaOH buffer pH 8.2
3 g	Na-tiosulfate (solid)
15 g	Difco Bacto-agar

Trace Mineral (1L)	
2.86 g	H3BO3
1.81 g	MnCl2 (4 H20)
0.222 g	ZnSO4 (7 H20)
0.39 g	Na2MoO4 (2 H2O)
0.079 g	CuSO4 (5 H2O)
0.0494 g	Co(NO3)2 (6 H2O)

Table 1: Recipes for BG-11 agar and liquid media

Cultures of each strain were viewed under light and fluorescent microscopy. Cyanobacteria 7942 are visible under light microscopy without staining and appear as light green rods (see Figure 19). Cyanobacteria 7942 fluoresce red as long bright rods (see Figure 20). During periods of rapid culture growth, when the culture tubes began to turn from a light green to a full, dark forest green color, cell division is apparent and visually looks similar to cellular filaments. Based on visual inspection of various different cultures with a variety of shades of green, the darker green the culture, the more dense the culture.

Figure 18: Image of cyanobacteria 7942 under light microscopy; 100x.

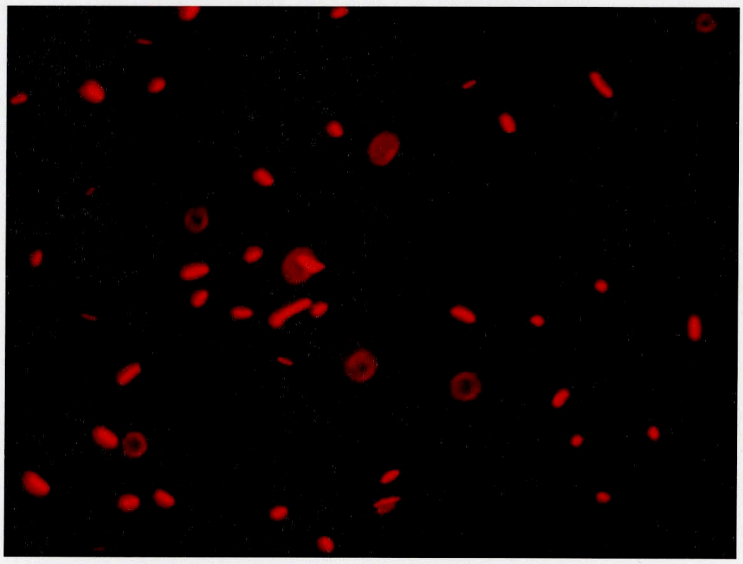


Figure 19: Image of cyanobacteria 7942 autofluorescence; 100x.

Cyanobacteria 6803, on the other hand, appear as cashew-shaped doublets (see Figures 21 & 22). Only rarely is one found alone. Potentially cyanobacteria 6803 either exist as doublets or the doublets are the result of cell division. Most evidence, though, appears to support the theory that cyano 6803 exist as doublets. The cyano 6803 have, in general, a duller green color throughout the culturing processes, and the cultures also change from a light green or clear at innoculation to a dark army green after growth.

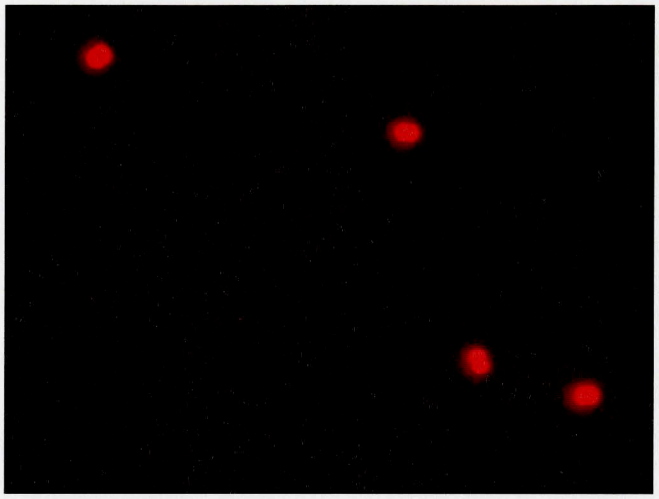


Figure 20: Image of cyanobacteria 6803 doublets and their red autofluorescence; 100x.

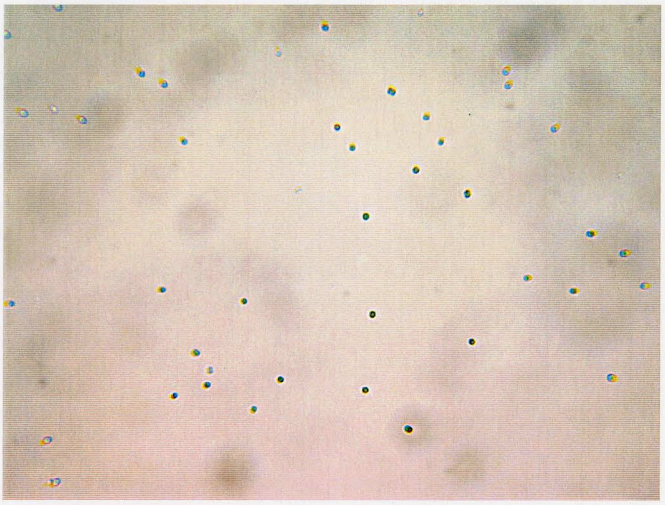


Figure 21: Image of cyanobacteria 6803 doublets using light microscopy; 40x.

In our facility, cyanobacteria were grown at ambient room temperature, typically around 23C, with anywhere between 2 & 4 degree variations. Both cultures, but particularly the 6803 strain grew

slowly, taking as long as a week to reach a full dark green color when started from another liquid culture. When starting from cells on a plate, growth may take as long as two weeks to become a visible green color. Liquid cultures were 50 ml in a 125 ml erlenmeyer flask, 100 ml in a 250 ml erlenmeyer flask or 200 ml in a 500 ml erlenmeyer flask. All growth was done under fluorescent lamps and shaking at roughly 225 rpm in a culture hood. Cultures grew equally well in all flasks. Using starter cultures, in 15 ml culture tubes, were less successful than starting cultures directly in 125 ml flasks, so starter cultures were abandoned early on. An interesting feature of cyanobacteria identified during our experience growing them was their hardiness. Both strains of cyanobacteria are able to withstand extreme lengths of time without additional media or treatment of any kind. In fact, we have left 50 ml cultures in 125 ml erlenmeyer flasks stagnant for over 6 months and have successfully started new cyanobacteria cultures of both strain 7942 and 6803; these cultures grew and had identical morphology under light microscopy.

The ability of cyanobacteria to survive long periods of time with few nutrients was a particularly useful characteristic during a major contamination of cyano supplies. We were even able to co-culture the two strains of cyanobacteria successfully (see Figure 23). Due to difficulties in maintaining cyanobacteria 7942 and 6803 on plates and no abil-

ity yet to regrow frozen cultures, the entire stock of cyanobacteria ended up contaminated with another microbe.

Gram staining of the milky green cultures identified small gram-positive bacteria around the cyano bacteria. The cyanobacteria 7942 had a more morphologically distinct interaction with the gram-positive contaminants (see Figure 24). These unidentified gram-positive spherical bacteria aggregate around the long cyanobacteria strain 7942 rods, eventually leading to the death of the cyanobacteria. Images of cyanobacteria 6803,

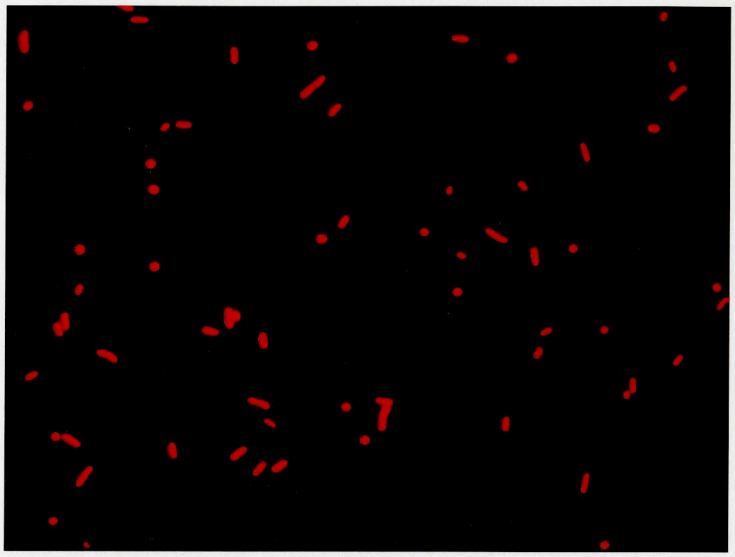


Figure 22: Image of cyanobacteria 7942 & 6803 co-culture; 100x.

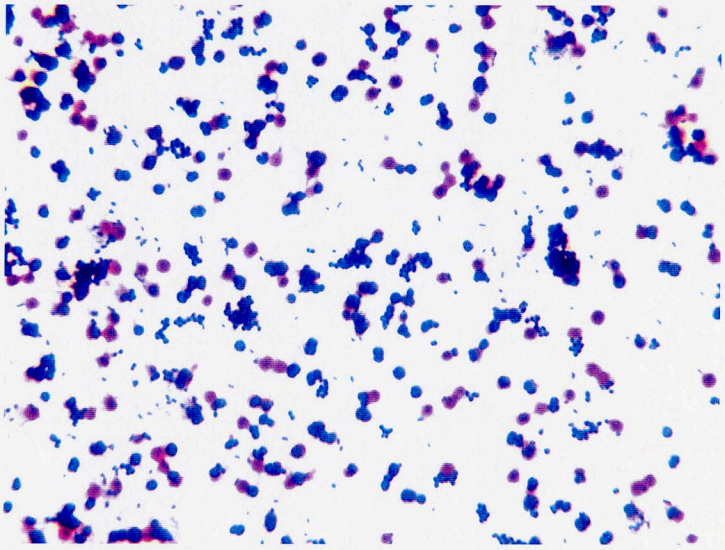


Figure 23: Image of gram stained culture containing both cyanobacteria 6803 and the gram-positive contaminant; 100x.

while not as definitive, did indicate a similar mechanism of attack and overgrowth (See Figure 25).

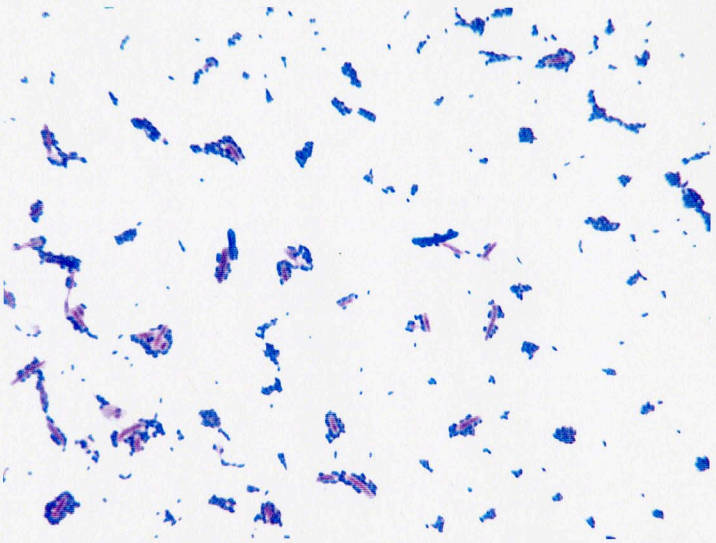


Figure 24: Image of gram stained culture containing both cyanobacteria 7942 and the gram-positive contaminant; 100x.

Starting new cultures in media without glucose proved advantageous for the cyanobacteria. After a few weeks away from glucose, the gram-positive spheres died off and only the cyanobacteria survived and continued to grow. Even though all cultures were contaminated initially, pure samples of both strains were successfully isolated through the starvation technique. This evidence for the rugged ability of cyanobacteria to survive harsh conditions for long periods of time lends credence to the notion that cyanobacteria are the best host for a modular B-MAD system, as it adds stability and another flexibility for application environments.

V. harveyi BB170 Reporter Strain Growth

A reporter strain of V. harveyi, BB170, senses AI-2 and responds naturally to the signal with bioluminescence but lacks the machinery to produce the intercellular signaling molecule [20]. The AI-2 reporter bug is an important assay tool for the construction of the Blue Light Converter and ACC cells. There is also the possibility that AI-2 from another, simpler to generate, source such as E. coli will be incorporated as an AI-2 generator for assay material to use during the development of the varying components to the B-MAD system.

V. harveyi is a comma-shaped, gram-negative marine bacterium. It grows in AB Media. We obtained the recipe for AB Media from Bassler at Princeton (see Figure 26). When using the specified AB media, V. harveyi strain BB170 grows in cultures in 1 to 2 days at ambient room temperatures. It was grown shaking at roughly 225 rpm in erlenmeyer flasks. V. harveyi grew successfully in every configuration of culture size, from small starter cultures right through to 1 liter cultures. Even though V. harveyi strain BB170 lacks the ability to produce AI-2 or VAI and thus cannot bioluminesce, a dim glow can be observed

AB Media	
17.53 g	NaCl
6.02 g	MgSO4
2.0 g	Cassamino Acids (vitamin free)
960 ml	distilled H20
*pH to 7.0 w/KOH. Autoclave	
10 ml	1M potassium phosphate, pH 7.0
10 ml	0.1M L-arginine
20 ml	50% glycerol

Table 2: AB Media recipe for culture of Vibrio harveyi.

from the larger cultures (1 liter) in a darkroom with all of the lights off. This level of bioluminescence would then represent a baseline level to compare to responses to various experimental systems where AI-2 is produced.

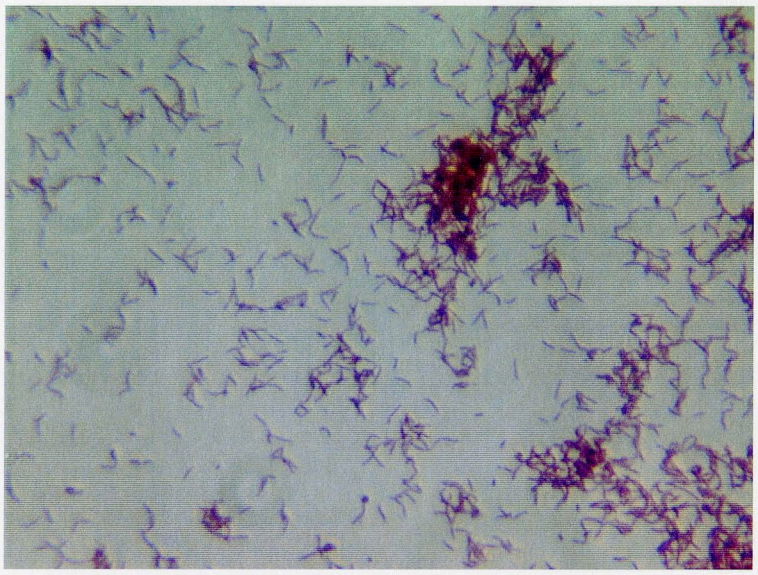


Figure 25: Image of the comma-shaped, gram-stained Vibrio harveyi strain BB170; 40x.

For verification and completeness, some V. harveyi were gram stained (see Figure27) and others were stained with BBI, a fluorescent stain (see Figure 28). The stains provided clear evidence of the cell morphology. The comma shape is apparent in the stains. The large cell densities that

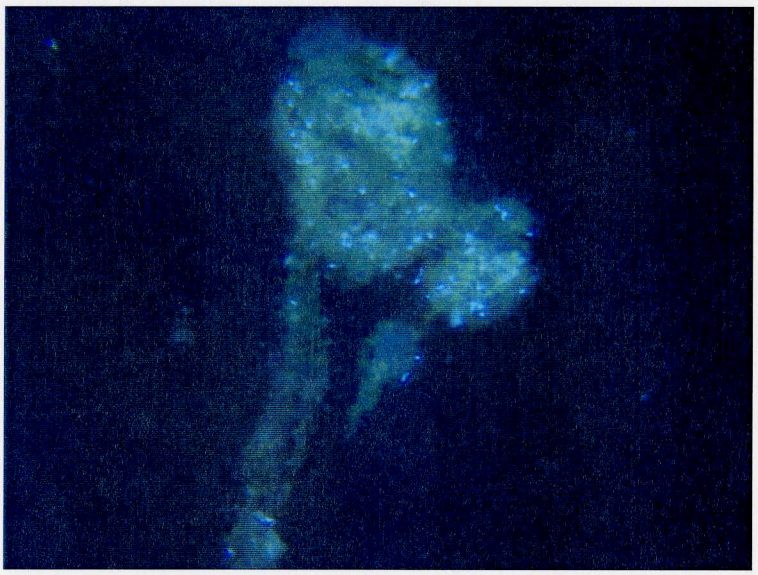


Figure 26: Image of V. harveyi BB170 under fluorescence after BBI staining; 40x. Small bright spots are V. harveyi BB170.

grow rapidly in the cultures is also evident, especially in the gram stain, which was taken from a culture less than a week old. An interesting observation of V. harveyi is their tendency to aggregate in the cultures, which is often still visible in the staining. In growing cultures, it was not uncommon to notice cloudy regions. These regions of the culture, when gently shaking the cultures in one's hand appear to look similar to a cotton-ball type consistency, with the entire culture being a milky white color. The media itself is completely clear, so it is easy to monitor growth of the bacteria. V. harveyi stran BB170 will prove and excellent assay tool because of its ease of growth and simply assay procedure for detection of AI-2. V. harveyi BB170 has successfully been frozen and stored. Testing has demonstrated that the bacteria can easily be re-cultured from frozen stock in starter culture tubes (15 ml) with a few ml of media.

Component Construction for AI-2 Blue Light Converter

The AI-2 Blue Light Converter cell is the first step towards building the final BLC. In actuality, the only difference between the AI-2 Blue Light Converter and the final B-MAD Blue Light Converter is one gene. The steps to create the AI-2 Blue Light Converter are broken away from the steps required to create the final Blue Light Converter. The AI-2 BLC converter is, in reality, nothing more than a cyanobacteria strain 7942 transformed with a plasmid containing the hliA gene linked to the luxS gene (see Figure 29), which codes for an enzyme used to produce AI-2. The addition of the pBLC_AI plasmid to cyanobacteria 7942 will create the AI-2 BLC.

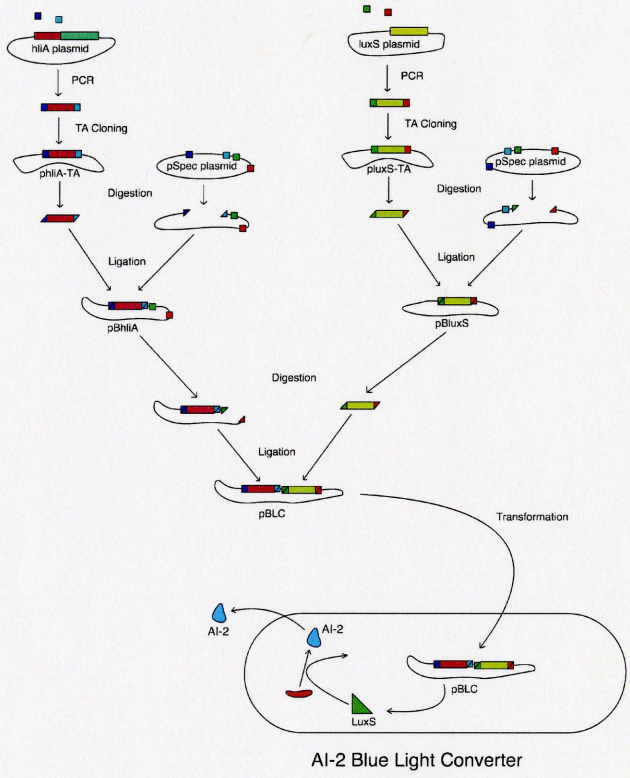


Figure 27: Illustration of construction outline for the AI-2 Blue Light Converter.

The first step in the construction of the BLC is to choose a host plasmid to build in the required genetic components. For the AI-2 BLC (and actually the final BLC too),

the pSpec plasmid from Vermaas at Arizona State University is the backbone (see appendix A for sequence). The pSpec plasmid contains two antibiotic resistance genes, ampicillin and spectinomycin. The pSpec plasmid also has a enough unique restriction enzyme sites to accomodate cloning multiple genes into the plasmid. The pSpec plasmid initially received from the Vermaas lab was transformed into chemically competant subcloning efficiency DH5 alpha cells and grown. This vector has been successfully used in transformation of cyanobacteria 6803.

The promoter for the hliA gene was acquired from van Waasbergen at the University of Texas Arlington. To clone the hliA promoter, the sequence of the promoter and hliA gene were used to design sequencing primers for the phlip plasmid received from van Waasbergen, as the sequence for the entire phlip plasmid was unavailable. Since the only portion of the phlip plasmid, which contains the hliA promoter, that we were interested in was the hliA promoter region, we designed sequencing primers from the sequence of the plasmid. These primers allowed us to successfully confirm the sequence of the promoter region as well as give interesting insight into the plasmid. The gene attached to the promoter on the provided plasmid was not, in fact, the hliA gene (see appendix A for 930 BP sequence successfully obtained from sequencing primers). As a result, sequencing primers designed based on the hliA gene sequence were unsuccessful. Confirming the sequence of the phlip plasmid made it possible to plan for sub-cloning the promoter into the pSpec plasmid.

Evaluating the restriction map of pSpec generated on the New England Biolabs website Nebcutter 2,0 utility, one unique restriction enzyme site, Ndel, and one two-cutter restriction enzyme site, BamHI, were chosen for the insertion site (see Figure 30). BamHI was chosen because it is a robust, common and easy restriction enzyme to work with. When cutting with the BamHI enzyme, which cuts at bp 2419 and bp 4193, the spectinomycin resistance gene is lost. However, both multi-cloning regions remain, as does the ampicillin resistance gene. The Ndel enzyme site is a few hundred base pairs early, at 2186 bp. Neither enzyme site occurs in the hliA promoter sequence being cloned out.

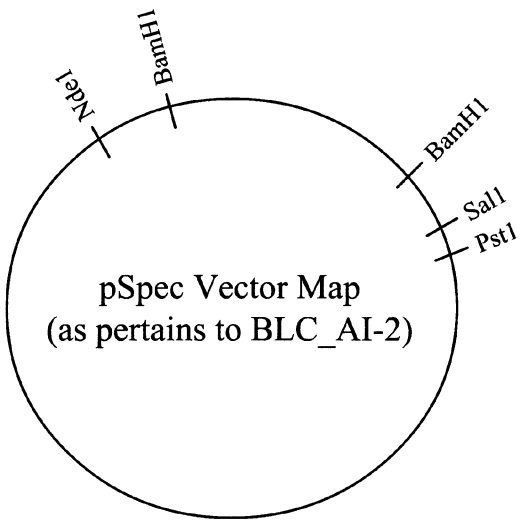


Figure 28: pSpec vector map with pertinent enzyme sites for the BLC_AI-2

As there are not convenient enzyme sites of any kind, not limited to the Ndel and BamH1 chosen, at the beginning or end of the hliA promoter sequence, the desired restriction enzyme sites will be designed into the PCR primers. Additional spare base pairs are added to the end of the primer, on the opposite side from the hliA promoter se-

quence, to ensure enzyme stability. The PCR primer sequence can be found in Appendix B. The PCR setup (see Figures 31 & 33) was run with the intention of TA cloning using Invitrogen's TA cloning kit and the pCR2.1 coning vector. TA cloning is a mechanism used to clone PCR products directly into a vector (see Figure 32). PCR leaves extra adenine bases on the ends of the PCR products. The TA cloning vectors have thymidine overhangs. These thymidine overhangs bind the adenine overhangs on the PCR product during ligation, closing the vector.

Dion TA PCR Program	
1	95 deg. For 15 min.
2	95 deg for 1:30
3	57 deg for 0:50
4	72 deg for 1:30
5	24 times to step 2
6	57 deg for 0:50
7	72 deg fro 5:00
8	Hold at 4 deg

Table 3: Description of PCR program used for PCR prior to TA Cloning.

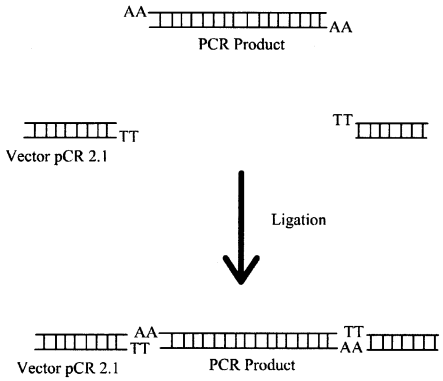


Figure 29: Illustration of TA Cloning.

The PCR program for PCR for the TA cloning of the hliA promoter with a NdeI restriction enzyme site at the 5′ end and a BamHI site at the 3′ end of the DNA is titled DionTA. The specific number of cycles required in the program was optimized through work conducted on earlier PCR of various DNA.

Immediately after the PCR reaction finished, a portion of the sample was run out on a gel for confirmation prior to cloning. A 1kb DNA ladder was the reference for the gel. The PCR came out successful, with the roughly 500bp band visible in a DNA gel (see figure 34). However, there was a bright band at the bottom of the gel. This band corresponds to the overloading of primer in the reaction mixture. Prior to adding the primers, the 100uM stocks are diluted by 10. In this case, the dilution was skipped, but the PCR worked OK; proceeding with the TA cloning would be fine.

hliA TA Cloning Rxn	
10x PCR buff.	10 ul
2.5 mM dntps	10 ul
8hr primer	10 ul
ndf primer	10 ul
Taq polymerase	1 ul
hliA dna (0.01ug)	5.55 ul
dH20	53.45 ul
Total rxn vol.	100 ul

Table 4: Description of TA Cloning reaction setup.

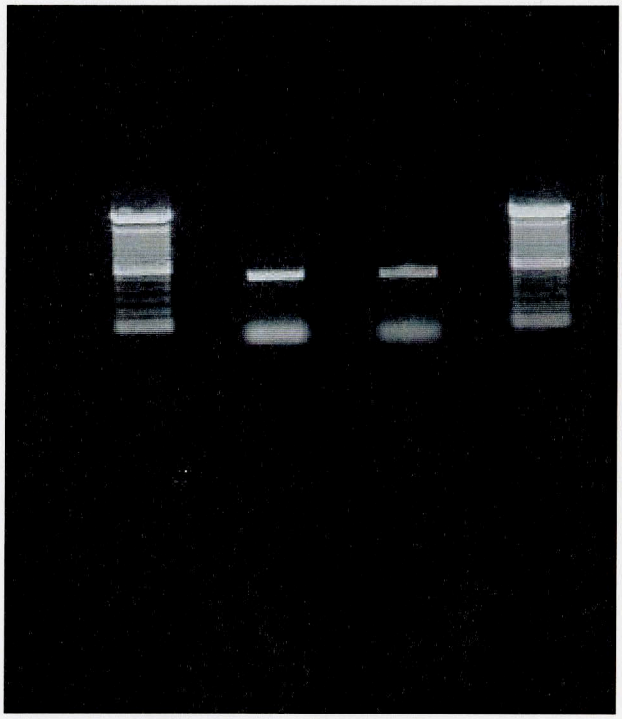


Figure 30: Gel Image from hliA TA cloning PCR confirming successful PCR of the roughly 500bp hliA promoter; 100bp ladder ran for size comparison.

Three separate ligation reactions were setup for the hliA-TA cloning experiment. In reaction number one, 1 ul of the PCR product is directly added to the ligation reaction. In reac-

tion number two, 1 ul of PCR product is diluted 1:100, so that 6.4ng of DNA, approximately 1:1 vector to insert ratio, is used. In the 3rd TA cloning reaction for hliA, 3 ul of the 1:100 diluted PCR product is added to the reaction, yielding a vector to insert ration of approximately 1:3. The TA cloning reactions run were the recommended 10ul reaction protocol in the Invitrogen TA cloning kit. After transformation of the ligation reaction the following day, colonies of E. coli were seen on all incubated plates. Plasmid preparation of the colonies grown in liquid media using a Qiagen miniprep kit from the hliA-TA cloning were successful and had high yields of DNA. Having successfully created the hliA-TA plasmid, with the NdeI and BamHI sites now artificially flanking the hliA promoter, is possible to begin construction of the plasmid pBhliA.

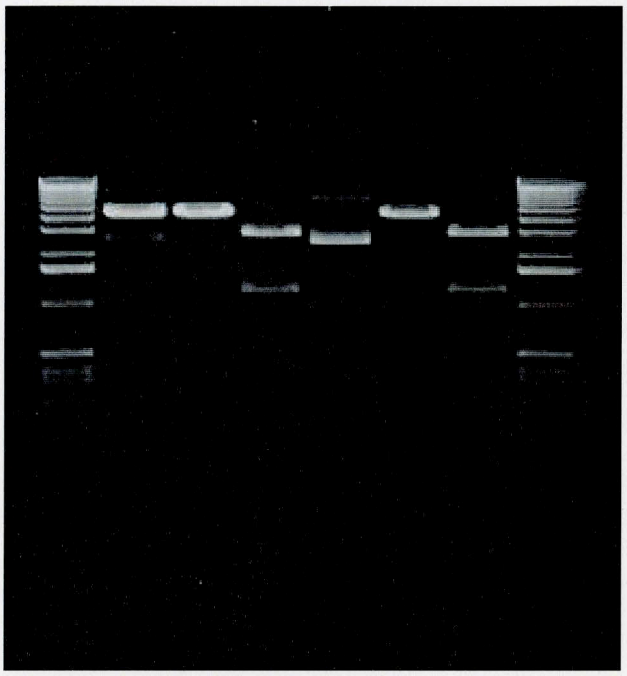


Figure 31: Gel image of enzyme efficiency test with a 1kb DNA ladder: lanes 1&4 are Nde1, lanes 2 &5 are PstI and lanes 3&6 are BamH1.

Prior to digestion of phliA-TA for further use in cloning, a quick check of enzyme efficacy was run on the phliA-TA plasmid. As there is also a PstI enzyme site on the pCR

2.1 cloning vector and the enzyme will be required in later experimentation with the vector, PstI is included in the study with BamH1 and NdeI. The clearly definied and clean bands seen in the resulting gel from the test enzyme digestion indicate, at least in single digestion, standard 4 hour digestions, BamHI, PstI and NdeI are all effective and work with the pCR 2.1 vector without surprises (see Figure 35).

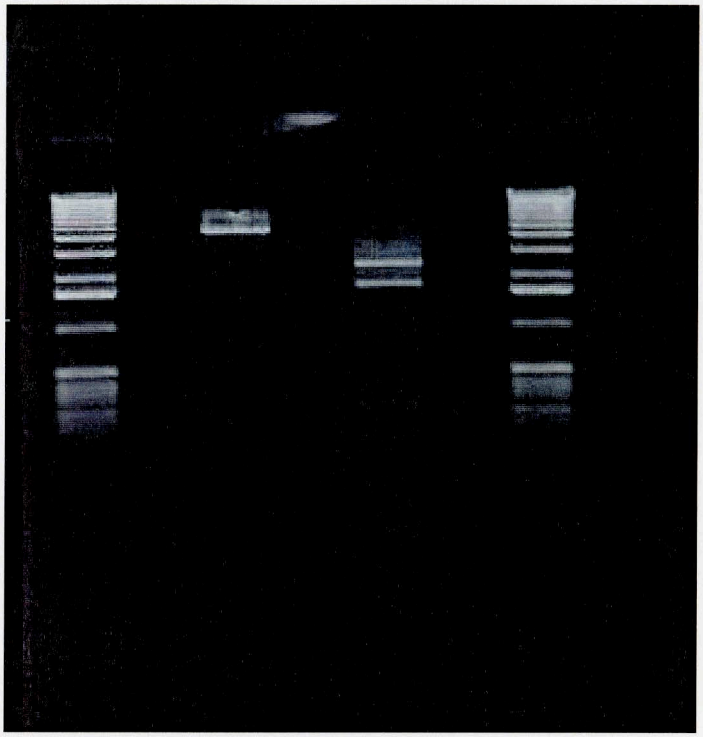


Figure 32: Gel image with NdeI & BamH1 digested pSpec vector illustrating the two distinct bands formed from the digestion (lane 4); 1kb DNA reference ladder.

Samples of pSpec grown in E. coli DH5 alpha cells and samples of phliA-TA grown in E. coli DH5 alpha were isolated using a Qiagen plasmid mini-prep kit for digestion and subsequent ligation. To create a pBhliA plasmid, the pSpec host vector must be digested using BamHI and NdeI to create a piece of linear DNA to accept the insert. Conversely, the hliA promoter needs to be digested out of the phliA-TA plasmid for insertion into the cut pSpec (see Figure 36). 20ul double digestion reactions with 1ul of each restriction enzyme used with the associated buffer to cut 1ug of plasmid

DNA. The digests ran for 4 hours. The pSpec digestion yields two (2) large linear segments of DNA. The smaller of the two is the insert between NdeI and the second BamHI (the first is in the middle and lost during digestion) is discarded. The larger of the inserts has both restriction enzyme ends, the other sections of the multi-cloning region and the ampicillin resistance gene. The hliA digestion came out streaked and varied (see Figure 37), while the pSpec digestions were clean, neat and identical. Further investigation implied that the difference between the two types of hliA-TA digests was a result of the efficacy of NdeI to cut. In the digestions that had a slightly larger size (a blip on the 100bp ladder run), the NdeI enzyme did not cut. Depending on the orientation of the insert in the TA cloning vector (PCR products insert randomly forwards or backwards into the TA cloning kits), an NdeI site sits close to a BamHI site on the pCR 2.1 cloning vector. Thus, since BamHI cuts well, the implication is that the NdeI was unsuccessful at cutting off the small piece

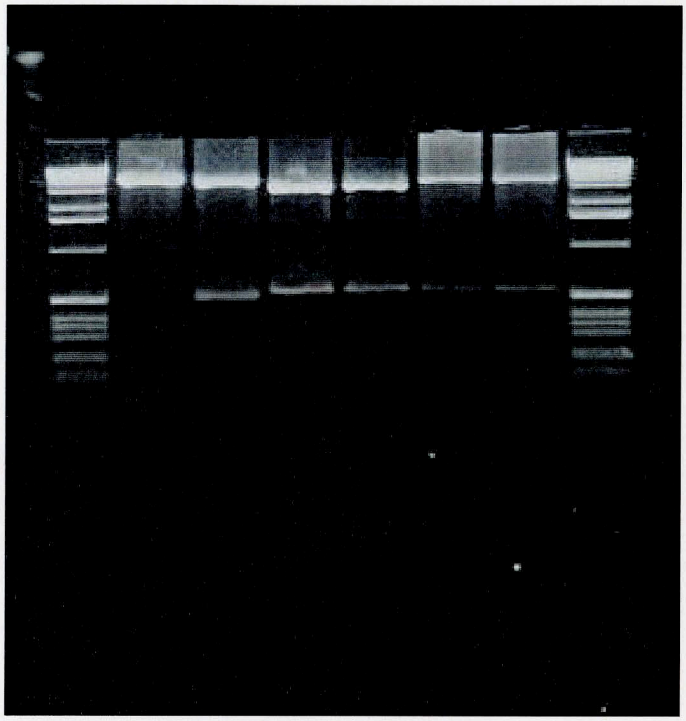


Figure 33: Gel image of three (3) hliA-TA Nde1 & BamH1 digestions; every 2 lanes are the same (1,2 are both from the same sample, 3,4 the same and 5.6 the same). 1kb DNA reference ladder.

left between BamHI and NdeI at the end of the hliA promoter in some instances. By selecting the smallest of the hliA-TA digested samples, the sample most likely contains successful NdeI-BamHI digested inserts (see Figure 39).

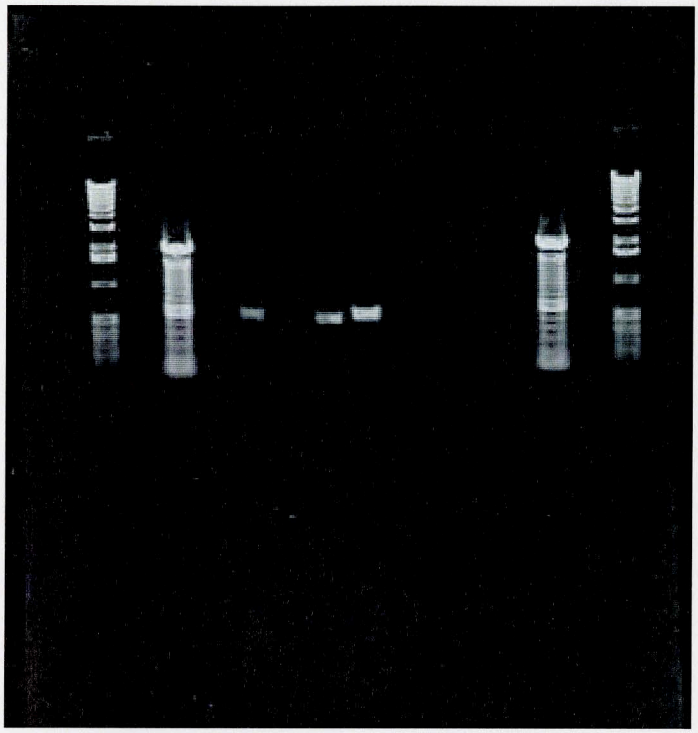


Figure 34: Gel image of 3 hliA NdeI and BamH1 digestions from Fig. 35 after gel extraction cleanup and 1 NdeI and BamH1 pSpec digestion (barely visible); 1kb DNA reference ladder in outermost lanes and 100bp ladder in inner lanes.

After digestion of the samples and running the samples out on gels, the desired bands were carefully cut out of the gel and run through a Qiagen gel extraction kit. Prior to ligation, a tiny portion of these extracted samples were run out on a gel to ensure the individual components for the ligation were clean and accurate, maximizing the chances for a successful ligation (see Figure 38). The difference in the hliA cuts is more clearly shown in the post-gel extraction gel, while the band for the cut pSpec is barely visible (see Figure 36). The brightness in the bands serves as a general indicator of the DNA content of the respective bands. In

these samples, the insert is far more concentrated than the pSpec vector.

The pBhliA ligation reaction is run in a similar setup to the TA-cloning ligation (see Figure 39). Here, there are three 10ul ligation reactions run for the pBhliA ligation. Each reaction contains 1ul of T4 DNA ligase and 1ul of ligation buffer provided with the T4 DNA ligase. In the first reaction, no insert is added to the reaction. Instead, there is 3ul of cut vector and plain water. This reaction is designed to indicate to serve as a negative indicator of the ligation reaction. If the vector is properly cut and clean, it will not ligate with itself because it does not have matching ends. However, if the ends happen to be identical, allowing the vector to ligate to itself, this first reaction will be a warning that

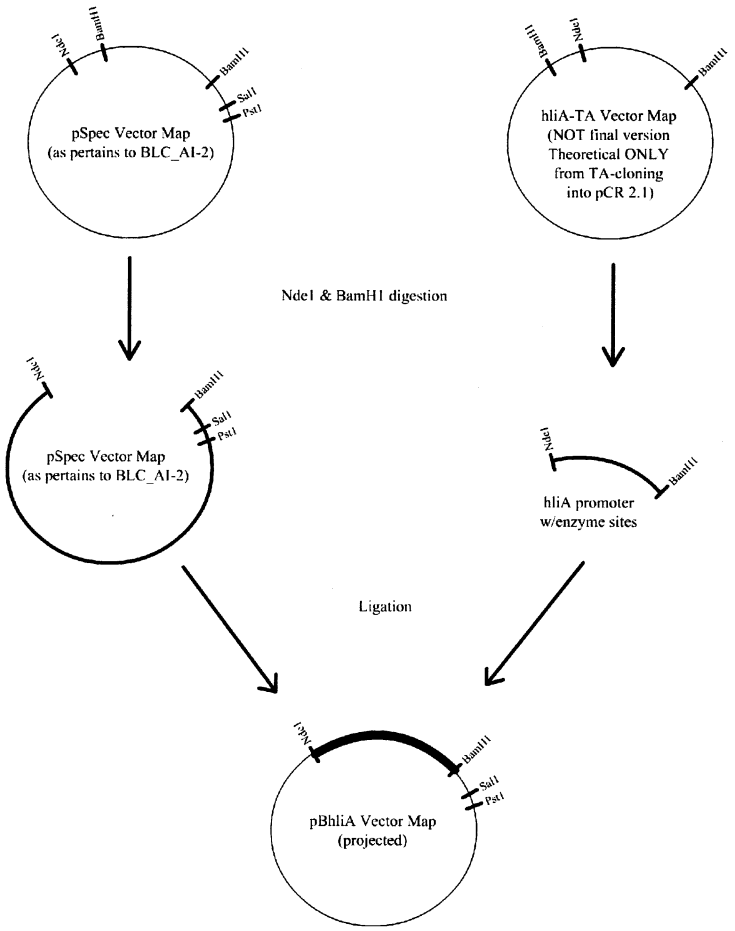


Figure 35: Illustration and vector diagrams for construction of pBhliA.

any colonies appearing in the other ligations may be incorrect. It can also serve as an effective percent indicator in the event that some of the vectors do ligate themselves together. In this case, the ratio of colonies from this reaction compared to the number of colonies in the other reactions indicate the general ratio of successful to unsuccessful ligated colonies on the plates. In the second ligation reaction, 3ul of the cut pSpec vector and 0.6ul of the cut vector are combined with the T4 DNA ligase, buffer and sterile water to make up the 10ul reaction. These amounts are supposed to be roughly a 1:1 vector to insert ratio. The third reaction is designed to be a 1:3 vector to insert ratio and provide an additional chance for ligation, as sometimes if ligations do not work at a 1:1 vector ratio, they may work at a 1:3 vector to insert ratio. In this reaction, 3ul of cut pSpec was combined with 1.8ul of the cut hliA promoter, T4 DNA ligase, buffer and sterile water to make up the 10ul reaction. The ligation reactions were left overnight in a thermocycler set to 14 deg C.

The ligation results were clear. After transforming max efficiency DH5 alpha chemically competent cells from Invitrogen, the plates incubated overnight. One plate was inoculated with 200ul of the transformation solution. The second plate was inoculated with any bacteria remaining on the wand used to spread the 200ul in the first plate, increasing the chances of isolated colonies in the event of large transformation success. The plates from the first reaction yielded, as expected, no colonies, meaning that the vector should not re-ligate to itself. The second ligation reaction, intended to be a 1:1 vector

to insert ratio, yielded 2 visible colonies. The third reaction, designed to be approximately a 1:3 vector to insert ration, yielded 4 visible colonies. As anticipated, the third reaction, at a 1:3 vector to insert ratio, had the largest yield. All of these were plated out again and then grown in liquid media and the plasmid pBhliA was isolated through a Qiagen plasmid miniprep kit. One sample was submitted to sequencing.

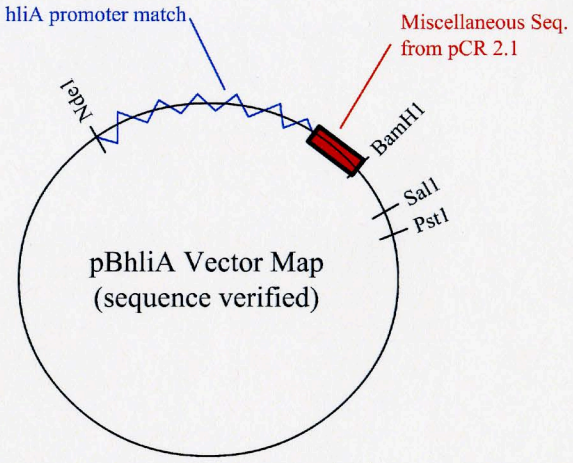


Figure 36: Sequence verified vector map for pBhliA.

The sequencing results from the submitted sample of pBhliA shows successful ligation of the hliA promoter and the pSpec vector. A detailed sequence, including color-coded regions of the DNA, can be found in Appenix A (see Figure 40 for a vector map). The sequence did not match exactly as expected. At the beginning of the sequence, there are some discrepancies in the first 13bp. These differences can easily be explained by looking at the graphical view of the sequence results (see Appendix A, pBhliA sequence). The dispute in this region is between double and triple nucleotides, and since the peaks are rounded and

long instead of sharp, it is plausible and very likely that in fact the first 13bp sequenced do in fact match the actual hliA promoter sequence perfectly. The other difference is towards the end of the promoter region. There is some additional DNA that at first did not match anything know at the end of the matching part of the hliA promoter and before the beginning of the pSpec plasmid. Careful inspection of this region shows that this DNA sequence is actually part of the pCR 2.1 TA cloning vector. Looking at the sequence of the pCR 2.1 vector, the 35 base pair region of DNA is located between the native BamHI restriction enzyme site in the pCR 2.1 vector and the insertion site (see Figure 41). For this strand of DNA to at the end of the insert region, the insert must have been cloned into the TA cloning vector in reverse (see Figure 3x). Additionally, a short section of DNA at the end of the hliA promoter, including the PCR primer and the artificially added enzyme site, is missing. Instead, the DNA ends in the sequence of DNA from the TA cloning vector. This phenomenon of losing the reverse PCR primer and associated DNA during TA cloning and then ligation to pSpec with the addition of a strand of DNA from the pCR 2.1 vector remains unexplained. However,

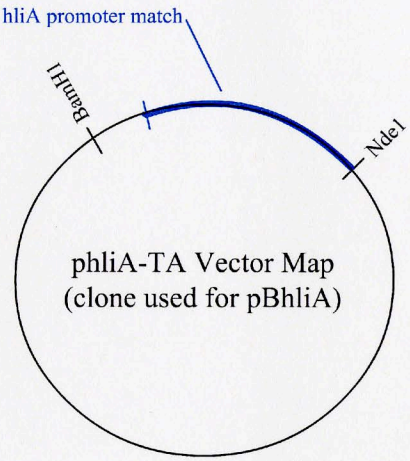


Figure 37: Vector map of phliA-TA used to create pBhliA.

given that only a small portion of the hliA promoter DNA is missing, albeit close to the ATG site, the constructed pBhliA should be an effective plasmid to use in the creation of the Blue Light Converter Cell for both AI-2 and C-6-HSL responses.

The next task in creating the blue light converter plasmid for AI-2 signaling is the incorporation of the luxS gene into the pBhliA or pSpec plasmid. First, the luxS plasmid on an unidentified plasmid with no known sequence, pBB2438, obtained from Bonnie Bassler at Princeton University, needs to be TA-cloned from the unknown plasmid with appropriate restriction enzyme sites flanking the gene.

Evaluating the restriction enzyme sites on the pSpec plasmid and considering the luxS sequence and the enzyme sites being removed by the cloning of the hliA promoter into the pSpec vector, SalI and PstI, at locations 4205bp and 4215bp in the pSpec sequence, respectively, were chosen as the enzymes to use in the cloning of luxS into the pSpec and/or pBhliA plasmid. As was the case with the hliA-TA cloning and associated PCR, the PCR primers are designed with the restriction enzyme sites artificially added to either end of the gene, in this case luxS, with a few additional base pairs added for stability. Dion-TA was once again

LuxS TA Cloning Rxn	
10x PCR buff.	10 ul
2.5 mM dntps	10 ul
SalI primer	1 ul
PstI primer	1 ul
Taq polymerase	1 ul
luxS dna	2 ul
dH20	75 ul
Total rxn vol.	100 ul

Table 5: Description of TA cloning reaction for the luxS gene.

the PCR program used for the PCR of luxS for TA cloning. The program parameters remained the same as used in the hliA-TA cloning.

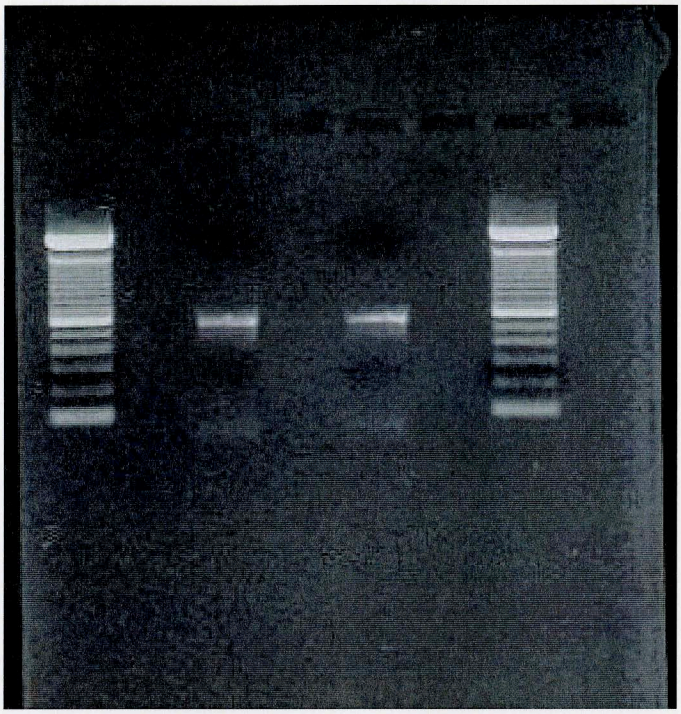


Figure 38: Gel image of the results of the luxS PCR for TA cloning; the band at roughly 525bp, size of the luxS gene, confirms the successful PCR. 100bp DNA reference ladder.

A sample from the PCR reaction was run out on a gel to determine whether or not the PCR reaction worked. The resulting gel showed successful PCR (see Figure 43). The bright band representing the cloned luxS gene with newly attached restriction enzyme sites was at the correct location in the 100bp ladder. The samples were then im-

mediately prepared for ligation in the Invitrogen TA cloning kit with the pCR 2.1 cloning vector.

For the TA cloning ligation reaction, the same setup was used that was used in the pBhliA and hliA-TA cloning reactions. Three samples were run in 10ul reactions. The first sample, a had no insert and only vector. The second sample had a 1:1 vector to insert ratio, and the third reaction had a 1:3 vector to insert ratio. The ligation and transformation were successful, as the incubated plates for vector and insert had colonies, while the plates for vector alone had no colonies. These colonies were then plated out and grown in liquid media. Plasmids were isolated using a Qiagen plasmid miniprep kit, with good yield of DNA from the plasmid isolation kit.

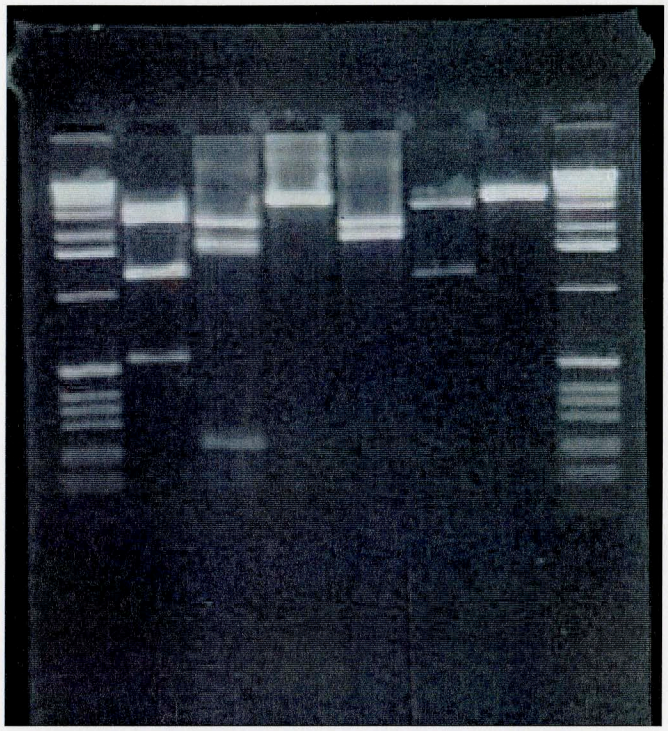


Figure 39: Gel image illustrating the double digestion of pluxS-TA plasmid (first sample DNA lane). The roughly 525bp land corresponds to the luxS gene cloned into the vector. 1kb DNA reference ladder.

The pluxS-TA plasmid resulting from the TA cloning was evaluated sequentially and through traditional molecular biology techniques. A double digestion of the pluxS-TA plasmid SalI and PstI, was done for confirmation (see Figure 44). The first column,

the only pluxS-TA sample run in the gel, had a gel band close to the 500bp band in the 1kb ladder, indicating that likely luxS is present in the sample. The sample was then sent for sequence analysis.

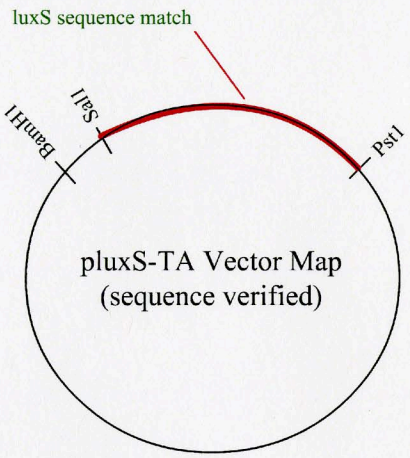


Figure 40: Sequence verified vector map of pluxS-TA.

Sequence analysis of plasmid pluxS-TA showed definitively that luxS and its associated restriction enzyme sites are successfully cloned into the pCR 2.1 cloning vector. See Appendix A for the specific sequence (see Figure 45 for vector map). The luxS sequences matched the known luxS sequence at every base pair. Additionally, the primers sequenced portions of the pCR 2.1 vector, and the vector matched perfectly to the sequence surrounding the luxS gene. Now that the pluxS-TA sequence has been confirmed, it is possible to continue with development of the AI-2 Blue Light Converter Plasmid.

At this stage, additional cloning is required to create the pBLC for AI-2 prior to the transformation of cyanobacteria and the first creation of the first Blue Light Converter Cell. Specifically, the luxS gene must be digested out of the pCR 2.1 cloning plasmid and into the pBhliA or into the pSpec first and then have the hliA promoter cloned

in the resulting plasmid. Once the luxS gene and hliA promoter are linked in a plasmid and sequence verified, it will be possible to finish the first Blue Light Converter. While tedious, the experimental evidence indicates that a Blue Light Converter, and the B-MAD system in general, is possible with additional molecular biology work.

Appendices

Appendix A: Sequences

pSpec Sequence:

TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTAT

CAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAG

AACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGG

CGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA

GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCG

TGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGA

AGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTC

CAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA

CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGG

TAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC

TAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC

TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGT

TTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTG

ATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCA

TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATC

AATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA

CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATA

ACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCA

CGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAG

AAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA

GTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGG

TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGT

TACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTC

AGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTA

CTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTG

AGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGC

GCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACT

CTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGA

TCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAAT

GCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTT

CAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT

TAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTC

GTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGG

TCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCG

GGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGA

GTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGG

CGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG

CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCA

GGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGG

GGATCCGCATGCCCGTTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGA

AAACCGAGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACG

GCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGA

AGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTC

GCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCAC

ACCGTGGAAACGGATGAAGGCACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGC

TGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG

GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGGGGTACAGTCT

ATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGA

GCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATGAGG

GAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGC

CATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTG

AAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACG

CGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATT

CTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAG

CTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCG

AGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCG

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TGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGA

GCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGC

GCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCG

TCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCG

CAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGC

AAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGC

GTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTAT

TATTTTTAAGCGTGCATAATAAGCCCTACACAAATTGGGAGATATATCATGAAAGGCTGGC

TTTTTCTTGTTATCGCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCTAGCGAG

GGCTTTACTAAGCTGATCCGGTGGATGACCTTTTGAATGACCTTTAATAGATTATATTACTA

ATTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAAATTTTTTCACAAAACGGTTTA

CAAGCATAAAGCTTGCTCAATCAATCACCGGATCCTCTAGAGTCGACCTGCAGGCATGCA

AGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC

ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCT

AACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA

GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGC

phlip sequence from the forward sequencing primer (930 BP)

GCAACTATTGCGCAGGATCGGCAGTGGATTGCCCTGGAGTTGAATATCTGGCTGGGAGAA

GTTACGGGCCAAACTGCAGATCAGCTCGGGTCCCCGTAAGTCCCAACGCAGCTGGAAGA

TACTGCCTGCATCCGCTTGGGTCAACGCGATCGCCGTATCCAGAAACAGGCTGAGGGCAG

CATCCGTGCTGTGTAGAGCGATCAGGGATTCCCGAAACTGAGTCAGTGGGTTTTCGGCGG

GATTGAGAGGGAGCGTCATCGCAGGCATGAGGAAGGCGATCGCTGCGCTCAGTCGAGGA

GGCGCAAGCAGTCGCAGCTTACCGCCAGCGGCGAGAAATCGCAGTAGGGATCAGGCTTA

GGGACTGACTGGGCAGAAAAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTAC

ACTTCAAACAGAACAACCAACCAAAAGGAATTCATTATGCGTAGCGGACGGATCCCCGG

GTACGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACT

CGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTG

GGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTT

CGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATA

CCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCATTACGGC

AAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCAGGGCGGCTATACGCCATTTGAA

GCCGATGTCACGCCGTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTGAACA

ACGAACTGAACTGGCAGACTATCCCGCCNGGGA

hliA promoter sequence (confirmed via sequencing)

GGGAACATTCAGCACTTCGCCAGTAGTCGCCACGTAGCCCGCCAAACTATTGCGCAGGAT

CGGCAGTGGATTGCCCTGGAGTTGAATATCTGGCTGGGAGAAGTTACGGGCCAAACTGCA

GATCAGCTCGGGTCCCCGTAAGTCCCAACGCAGCTGGAAGATACTGCCTGCATCCGCTTG

GGTCAACGCGATCGCCGTATCCAGAAACAGGCTGAGGGCAGCATCCGTGCTGTGTAGAG

CGATCAGGGATTCCCGAAACTGAGTCAGTGGGTTTTCGGCGGGATTGAGAGGGAGCGTC

ATCGCAGGCATGAGGAAGGCGATCGCTGCGCTCAGTCGAGGAGGCGCAAGCAGTCGCAG

CTTACCGCCAGCGGCGAGAAATCGCAGTAGGGATCAGGCTTAGGGACTGACTGGGCAGA

AAAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTACACTTCAAACAGAACAAC

CAACCAAAAGGAATTCATT

pBhliA sequence:

The red portion of the vector matches the insert. The black portion at the beginning is close to the hliA promoter sequence, however the signal is unclear here from sequencing. The green portion matches the pSpec host vector. The black section in the middle reflects a portion of the pCR 2.1 vector. The hliA promoter, had inserted backwards in the pCR 2.1, and that is the BamH1 site used in the cut.

GCACGTAGCCGCCAACTATTGCGCAGGATCGGCAGTGGATTGCCCTGGAGTTGAATATCTGGCT
GGGAGAAGTTACGGGCCAAACTGCAGATCAGCTCGGGTCCCCGNTAAGTCCCAACGCAGCTG
GAAGATACTGCCTGCATCCGCTTGGGTCAACGCGATCGCCGTATCCAGAAACAGGCTGAGGGC
AGCATCCGTGCTGTGTAGAGCGATCAGGGATTCCCGAAACTGAGTCAGTGGGTTTTCGGCGGG
ATTGAGAGGGAGCGTCATCGCAGGCATGAGGAAGGCGATCGCTGCGCTCAGTCGAGGAGGCG
CAAGCAGTCGCAGCTTACCGCCAGCGGCGAGAGATCGCAGTAAGGATCAGGCTTAGGGACTGA
CTGGGCAGAAAAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTACAAGCCGAATTCC
AGCACACTGGCGGCCGTTACTAGTGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC
GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAC
GAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC
GTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC
TGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATC
CACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGG
AACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACA
AAAATCGAC

pluxS-TA sequence:

ATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG

GCTTTGTGACGTCTTAGTCGATGCGTAGCTCTCTCAGCATTGACTCTGGCAGTGCCAATTC

ATCATTCTTATTCACCGCCACACCCACTTCTAGAATGTTCTTCGCGATTTGCTTCGCTTCATC

CAGAGAGTGCATCGCTGCTGTACCACATTGGTATTCGTTCAACTCAGGGATCTTGTTTTGGT

TTTCTACTTTTAGTACGTCTTCCATCGCGGCAATCCAAGCGTCAGCCACTTGCTGCTCTGAA

GGCGTACCAATCAAGCTCATGTAGAAACCAGTACGGCACCCCATTGGTGAGATATCAATG

ATCTCAACGCTATCACCATTTAGGTGATTACGCATAAAGCCTGCGTACAAATGCTCTAATGT

ATGAATTCCTTTCTCAGAAAGGATGTCTTTGTTTGGAGCAGTGAAACGTAGGTCGAATACC

GTGATGGTGTCTCCTTTTGGAGTTTGCATCGTTTTAGCCACACGAACCGCTGGTGCATTCAT

ACGCGTGTGGTCTACGGTAAAGCTGTCTAATAAAGGCATGTCGACCGCAGCCGAATTCCA

GCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGG

TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG

GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGT

TGCGCTCACTGCC

Appendix B: PCR Primers

PHLIP_front_rev:	CGA TTT GGC CCG TAA CTT CT
PHLIP_front_for :	CAT TCA GCA CTT CGC CAG TA
PHLIP_MID_for:	CCT CAA GTG GTT GTT GCT GA
PHLIP-f-r:	AGA TAT TCA ACT CCA GGG CAA TC
hliAprom3_for:	TCT CAT ATG GGG AAC ATT CAG CAC TTC GCC AGT AG
hliAprom2_rev:	GCG CCT AGG AAT GAA TTC CTT TTG GTT GTT G
luxS2_for:	GCG GTC GAC ATG CCT TTA TTA GAC AGC TTT ACC
luxS2_ reverse:	TGT GAC GTC TTA GTC GAT GCG TAG CTC TCT CAG

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